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ASSAY

This invention relates to a diagnostic method for the detection of a cytochrome *b* mutation in fungi that leads to strobilurin analogue resistance using the amplification refractory mutation system (ARMS). The invention also relates to mutation specific primers for use in the method and to diagnostic kits containing these primers.

Description

The widespread use of fungicides in agriculture is a relatively recent phenomenon, and most of the major developments have taken place during the last 40 years. Previously, farmers often ignored or did not recognise the effect that fungal pathogens had on the yield and quality of their crops. Nowadays, however, these losses are unacceptable, and farmers rely on the use of fungicidal chemicals to control fungal diseases. As a consequence, commercial fungicides have become an important component of the total agrochemical business, with world-wide sales in 1996 of about $5.9 billion, equivalent to 18.9% of the total agrochemical market (Wood Mackenzie, 1997a 'Agchem products- The key agrochemical product groups', in Agrochemical Service, Update of the Products Section, May 1997, 1-74). A large number of fungicides are already available to the farmer; in a recent edition of The Pesticide Manual (Tomlin, 1994 10th Edition, British Crop Protection Council, Farnham, UK, and the Royal Society of Chemisrty, Cambridge, UK) contains 158 different fungicidal active ingredients in current use. Nevertheless, further industrial research aimed at the discovery and development of new compounds is extremely intensive and product management procedures are extremely important in securing the best and longest lasting performance from fungicides with a particular mode of action and/or belonging to a particular compound series. In particular it is vital to develop effective resistance management strategies when fungicides with new modes of action are introduced.

The strobilurin analogues constitute a major new series of agricultural fungicides

mitochondrial respiration in fungi. More specifically, it has been established that these

mitochondrial membrane of fungi, by binding to a specific site on the cytochrome *b* protein. This family of inhibitors prevents electron transfer at the ubiquinone redox site Q_o on the dimeric cytochrome *b* protein (Esposti et al 1993 Biochim. et Biophys Acta 243-271). Unlike many mitochondrial proteins, the cytochrome *b* protein is mitochondrially encoded.

Reports in the literature show that key amino acid changes at the cytochrome *b* target site can affect the binding of strobilurin analogues. In depth mutagenesis studies in *Saccharomyces cerevisiae* (JP Rago et al 1989 J. Biol. Chem. 264, 14543-14548), mouse (Howell et al 1988 J. Mol. Biol. 203, 607-618), *Chlamydomonas reinhardtii* (Bennoun et al 1991 Genetics 127, 335-343) and *Rhodobacter spp* (Daldal et al 1989 EMBO J. 3951-3961) have been carried out. Interesting information was also gathered from studying the natural basis for resistance to strobilurin analogues in *Paracentrotus lividus* - Sea Urchin (Esposti et al 1990 FEBS 263, 245-247) and the Basidiomycete fungi *Mycena galopoda* and *Strobilurus tenacellus* (Kraiczy et al 1996 Eur. J. Biochem. 235, 54-63), both of which produce natural variants of the strobilurin analogues. There are two clear regions of the cytochrome *b* gene where amino acid changes have a dramatic effect on strobilurin analogue binding. These areas cover amino acid residues 125-148 and 250-295 (based on *S.cerevisiae* coding system). More precisely amino acid changes at residues 126, 129, 132, 133, 137, 142, 143, 147, 148, 256, 275 and 295 have been shown to give rise to resistance to strobilurin analogues (Brasseur et al 1996 Biochim. Biophys. Acta 1275, 61-69 and Esposti et al (1993) Biochimica et Biophysica Acta, 1143, 243-271).

In the present invention we have now devised novel diagnostic methods for the detection of a point mutation in a fungal cytochrome *b* gene based on ARMS (Amplification Refractory Mutagenesis System) wherein the mutation gives rise to resistance to a strobilurin analogue. These methods are suitable for the detection of this resistant genotype to any of the strobilurin analogues or any other compound in the same cross resistance group. Robust tests have been developed for the detection of this point mutation in a range of fungal plant pathogens. Compounds may be considered to be in the same cross resistance group when the resistance mechanism to one compound also confers resistance to another, even when the modes of action are not the same.

According to the first aspect of the invention we provide a method for detecting a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal

resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

The methods of the invention are particularly suitable for the detection of mutations in a fungal cytochrome *b* gene and most preferably wherein said mutation in the fungal cytochrome *b* gene results in one of the following amino acid substitutions: $A_{126}T$, $F_{129}L$, $Y_{132}C$, $C_{133}Y$, $G_{137}R/S/E/V$, $W_{142}T/K$, $G_{143}A$, $I_{147}F$, $T_{148}M$, $N_{256}Y/K/I$, $L_{275}F/S/T$ or $L_{295}F$, the presence of which have been found to give rise to fungal resistance to strobilurin analogues or any other compound in the same cross resistance group.

The strobilurin analogues and compounds in the same cross resistance group include for example, azoxystrobin, kresoxim-methyl, trifloxystrobin, famoxadone and fenamidone.

In a preferred embodiment of the first aspect of the invention we now provide a diagnostic method for the detection of a $G_{143}A$ mutation in a fungal cytochrome *b* gene, which method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the $G_{143}A$ mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when a $G_{143}A$ mutation is present in the sample; and detecting the presence or absence of the said $G_{143}A$ mutation by reference to the presence or absence of a diagnostic primer extension product.

As used herein the term $G_{143}A$ is used to denote the substitution of a glycine residue by an alanine residue in a fungal cytochrome *b* sequence at the equivalent of the position of the 143rd codon/amino acid of the *Saccharomyces cerevisiae* cytochrome *b* sequence. This nomenclature is used for all the other residues [illegible]

First position	Second position		Third position
5'end	C	G	3'end
G	Alanine	Glycine	U
	Alanine	Glycine	C
	Alanine	Glycine	A
	Alanine	Glycine	G

Table 1: codon usage

A glycine to alanine point mutation can only arise as a result of a G to a C change at the second base of the codon. Other mutations may also arise at the 3rd position in the codon due to complete degeneracy in genetic code for alanine and glycine (see table 1) but this is readily taken into consideration when designing the ARMS primer. (The concept of ARMS primers is described fully in Newton et al, Nucleic Acid Research 17 (7) 2503-2516 1989). As a result ARMS primers can be designed for the detection of the $G_{143}A$ point mutation given only sequence information on the wild type, strobilurin analogue sensitive, cytochrome *b* gene. There is no need to have access to a resistant isolate in new fungi of interest resulting from a $G_{143}A$ mutation. Some examples of relevant plant pathogenic fungi are listed in Table 3. This list is not meant to be in any way exclusive. The skilled plant pathologist will be able to readily identify those fungi to which this assay is relevant.

	Examples of species where $G_{143}A$ can be assayed for:
1	*Plasmopara viticola*
2	*Erysiphe graminis f.sp. tritici/hordei*
3	*Rhynchosporium secalis*
4	*Pyrenophora teres*
5	*Mycosphaerella graminicola*
6	*Venturia inaequalis*
7	*Mycosphaerella fijiensis* var. *difformis*
8	*Sphaerotheca fuliginea*

9	*Uncinula necator*
10	*Colletotrichum graminicola*
11	*Pythium aphanidermatum*
12	*Puccinia hordei*
13	*Puccinia recondita*
14	*Magnaporthe grisea*
15	*Phytophthora infestans*

Table 2: Example of species where $G_{143}A$ is assayable

The methods of the invention described herein are particularly useful in connection with plant pathogenic fungi and especially with the following fungal species : *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Puccinia hordei, Puccinia recondita, Magnaporthe grisea and Phytophthora infestans.*

The test sample of nucleic acid is conveniently a total DNA preparation from fungal material, a cDNA preparation from fungal material or the fungal material itself or plant or seed extracts containing fungal nucleic acid. In this invention, we describe the detection of the $G_{143}A$ mutation by using total DNA preparation, cDNA preparation and by directly using spore material as template in the PCR reactions. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample. That is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique such as PCR before use in the method of the invention.

Any convenient enzyme for polymerisation may be used provided that it does not

thermostable enzymes which have no significant 5' -> 3' exonuclease activity, for example Taq DNA polymerase, particularly "Ampli Taq Gold" ™ DNA polymerase (PE Applied

Biosystems), Stoffel fragment, or other appropriately N-terminal deleted modifications of Taq or Tth (*Thermus thermophilus*) DNA polymerases.

We have now devised primers for the $G_{143}A$ mutation in the above-listed fungal species which have been shown to detect the specific mutations reliably and robustly.

In a further aspect the invention therefore provides a diagnostic primer capable of binding to a template comprising a mutant type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds to a nucleotide present in said mutant form of a fungal cytochrome *b* gene and the presence of said nucleotide gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group.

The diagnostic primer of the invention is preferably at least 20 nucleotides in length, most preferably 26 nucleotides in length, but this may be between 15 and 20 nucleotides in length.

In a preferred embodiment of the above aspect of the invention the penultimate nucleotide (-2) of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence. In a further preferred embodiment the -3 nucleotide of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers capable of binding to a template comprising a mutant type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds to a nucleotide present in said mutant form of a fungal cytochrome *b* gene and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied with respect to the wild type sequence without significantly affecting the properties of the diagnostic primer.

In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers comprising the sequences given below and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given below and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer.

Conveniently, the sequence of the diagnostic primer is exactly as provided below. It is preferred that the ARMS primers in all aspects of the invention are 26 nucleotides in length. In all the primers listed below the penultimate nucleotide has been altered from wild type cyt *b* sequence to destabilise the primer thereby making it more selective for the desired template and these primers are particularly preferred according to the invention. It will be apparent to the man skilled in the art of primer design that bases alternative to or in addition to those discussed above may also be varied without adversely affecting the ability of the primer to bind to the template.

Primer #	species:	primer sequence for the detection of $G_{143}A$ (5' to 3')
1	*Plasmopara viticola*	CCTTGGTGACAAATGAGTTTTTGGAC
2	*Erysiphe graminis f.sp. tritici/hordei*	CCATACGGGCAGATGAGCCACTGGAC
3	*Rhynchosporium secalis*	CCTTATGGACAGATGTCTTTATGATC
4	*Pyrenophora teres*	CCCTACGGGCAAATGAGCCTTTGATC
5	*Mycosphaerella graminicola*	
6	*Venturia inaequalis*	
7	*Mycosphaerella fijiensis* var. *difformis*	
8	*Sphaerotheca fuliginea*	
9	*Uncinula necator*	
10	*Colletotrichum graminicola*	
11	*Pythium aphanidermatum*	
12	*Puccinia hordei*	
13	*Puccinia recondita*	
14	*Magnaporthe grisea*	
15	*Phytophthora infestans*	

Table 3: ARMS primer design for the detection of the $G_{143}A$ mutation

Such primers may be manufactured using any convenient method of synthesis

Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993, 1st Edition.

It will be appreciated that extension of a diagnostic primer can be designed by

In a further aspect the invention therefore provides a diagnostic primer capable of binding to a template comprising wild type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds to a nucleotide present in a wild type fungal cytochrome *b* gene said wild type fungus showing sensitivity to a strobilurin analogue or any other compound in the same cross resistance group.

In a preferred embodiment of this aspect of the invention the penultimate nucleotide of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence. In a further preferred embodiment the -3 nucleotide of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

The diagnostic primer of the invention is preferably at least 20 nucleotides in length, most preferably 26 nucleotides in length, but this may be between 15 and 20 nucleotides in length.

In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers capable of binding to a template comprising wild type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds to a nucleotide present in a wild type fungal cytochrome *b* and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied with respect to the wild type sequence without significantly affecting the properties of the diagnostic primer.

In a further particularly preferred embodiment of this aspect of the invention we provide diagnostic primers comprising the sequences given below and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given below and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer. Conveniently, the sequence of the diagnostic primer is exactly as provided below. In all the primers listed below the penultimate nucleotide has been altered from wild type cytochrome *b* sequence to destabilise the primer thereby making it more selective for the desired template. It will be apparent to the man skilled in the art of primer design that bases alternative to or in addition to those discussed above may also be varied without adversely affecting the ability of the primer to bind to the template.

Primer	Species	primer sequence for the detection of WT sequence (5' to 3')
1	*Plasmopara viticola*	CCTTGGTGACAAATGAGTTTTTGGAG
2	*Erysiphe graminis f.sp. tritici/hordei*	CCATACGGGCAGATGAGCCACTGGAG
3	*Rhynchosporium secalis*	CCTTATGGACAGATGTCTTTATGATG
4	*Pyrenophora teres*	CCCTACGGGCAAATGAGCCTTTGAAG
5	*Mycosphaerella graminicola*	
6	*Venturia inaequalis*	
7	*Mycosphaerella fijiensis* var. *difformis*	
8	*Sphaerotheca fuliginea*	
9	*Uncinula necator*	
10	*Colletotrichum graminicola*	
11	*Pythium aphanidermatum*	
12	*Puccinia hordei*	
13	*Puccinia recondita*	
14	*Magnaporthe grisea*	
15	*Phytophthora infestans*	

Table 4: ARMS primer design for the detection of the wild type sequence

The examples described above relate to ARMS primers based on the forward strand of DNA. The use of ARMS primers based on the forward strand of DNA is particularly preferred.

ARMS primers may also be based on the reverse strand of DNA if so desired. Such reverse strand primers are designed following the same principles above for forward strand primers namely, that the primers may be at least 20 nucleotides in length most preferably 26 nucleotides in length, but may be between 15 and 20 nucleotides in length and the final nucleotide at the 3' end of the primer matches the relevant template i.e. mutant or wild type

nucleotides in the primer may be varied without significantly affecting the properties of the diagnostic primer.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer in one or more cycles of PCR amplification. A convenient example of this aspect is set out in European patent number EP-B1-0332435. The further amplification primer is either a forward or a reverse common primer. For each species, the primer used is as below. The Primers 1-4 are reverse primers.

Primer	Species	primer sequence (5' to 3')
1	*Plasmopara viticola*	GATACCTAATGGATTATTTGAACCTACCT
2	*Erysiphe graminis f.sp. tritici/hordei*	AACACCTAAAGGATTACCAGATCCTGCAC
3	*Rhynchosporium secalis*	TACACCTAAAGGATTACCTGACCCTGCAC
4	*Pyrenophora teres*	TACACCTAAAGGATTTCCTGACCCTGCAA
5	*Mycosphaerella graminicola*	
6	*Venturia inaequalis*	
7	*Mycosphaerella fijiensis* var. *difformis*	
8	*Sphaerotheca fuliginea*	
9	*Uncinula necator*	
10	*Colletotrichum graminicola*	
11	*Pythium aphanidermatum*	
12	*Puccinia hordei*	
13	*Puccinia recondita*	
14	*Magnaporthe grisea*	
15	*Phytophthora infestans*	

Table 5: Primers to use with ARMS primers

A convenient control primer may be used which is designed upstream from the $G_{143}A$ position. When using these primers along with the corresponding reverse primer described above, amplification will occur regardless whether the $G_{143}A$ point mutation is present or not.

Primer	Species	Control primer sequence (5' to 3')
1	*Plasmopara viticola*	GCCTTGGGGACAAATGAGTTTTTG
2	*Erysiphe graminis f.sp. tritici/hordei*	GCCATACGGGCAGATGAGCCACTG
3	*Rhynchosporium secalis*	TCCTTATGGACAGATGTCTTTATG
4	*Pyrenophora teres*	ACCCTACGGGCAAATGAGCCTTTG
5	*Mycosphaerella graminicola*	

6	*Venturia inaequalis*	
7	*Mycosphaerella fijiensis* var. *difformis*	
8	*Sphaerotheca fuliginea*	
9	*Uncinula necator*	
10	*Colletotrichum graminicola*	
11	*Pythium aphanidermatum*	
12	*Puccinia hordei*	
13	*Puccinia recondita*	
14	*Magnaporthe grisea*	
15	*Phytophthora infestans*	

Table 5: Control primer design

A variety of methods may be used to detect the presence or absence of diagnostic primer extension products and/or amplification products. These will be apparent to the person skilled in the art of nucleic acid detection procedures. Preferred methods avoid the need for radiolabelled reagents. Particularly preferred detection methods are those based on fluorescence detection of the presence and/or absence of diagnostic primer extension products. Particular detection methods include "Scorpions"™ product detection as described in PCT application number PCT /GB98/03521 filed in the name of Zeneca Limited on 25 November 1998 the teachings of which are incorporated herein by reference, "Taqman" ™ product detection, for example as described in patent numbers US-A-5487972 & US-A-5210015; "Molecular Beacons" ® product detection, outlined in patent number WO-95/13399 and surface enhanced Raman resonance spectroscopy (SERRS), outlined in patent application WO 97/05280. Further preferred detection methods include ARMS linear extension (ALEX) and PCR with ALEX as described in published PCT application number WO 99/04037. Conveniently, real-time detection is employed. The use of "Scorpions"™ product detection as described in PCT application number PCT /GB98/03521 is particularly preferred for use in all aspects of the invention described herein. The combination of the

based detection method. Many of these detection methods are appropriate for quantitative work using all of the above primers. These different PCR

different tubes or multiplexed in one tube. Using such methods, estimates can be made on the frequency of point mutation molecules present in a background of wild type molecules.

As exemplified herein we have used ARMS primers based on the forward strand of DNA in combination with Scorpion primers based on the reverse strand of DNA as the detection method. The Scorpion primers preferably comprise the reverse primers shown in Table 5. It will be readily apparent to the man skilled in the art that alternative combinations of ARMS primers and Scorpion primers could also be used. For example the primer based on the forward strand of the DNA could be a combination of an ARMS primer with a Scorpion detection system and this could be used with a standard primer based on the reverse strand of DNA.

Genotypic testing of isolates enables the results of phenotypic bioassays to be related to the DNA profile of the target gene. The discovery of a single point mutation as the resistance mechanism explains the qualitative nature of the resistance, and the confirmation of single spore isolate sequences validates the accuracy of the screens in determining frequencies of resistant and sensitive isolates in the samples tested.

The development of the ARMS diagnostic method enables larger sample sizes to be analysed for the presence of the resistance mutation than would be feasible in a bioassay programme. Larger sample sizes enable the identification of the resistance mutation at frequencies of a lower percentage than might be easily detected through bioassay. This enables resistance to be identified in the population before it might be apparent from field data. The high throughput nature of the method enables a wider area and more sites to be sampled and tested than might be possible using the bioassay. ARMS diagnosis allows the detection of the presence of the resistance gene in a population before the effects of the gene can be viewed phenotypically by bioassay in heteroplasmic and/or heterokaryotic cells, thus reducing the error of classifying samples as sensitive when they carry a low frequency of the resistance genotype. Results are obtained much faster through simultaneous read-out compared to waiting for disease development *in planta*, enabling fast responses to field situations and advice on resistance management to be given more quickly.

One or more of the diagnostic primers of the invention may be conveniently packaged with instructions for use in the method of the invention and appropriate packaging and sold as a kit. The kits will conveniently include one or more of the following:

appropriate nucleotide triphosphate, for example dATP, dCTP, dGTP, dTTP, a suitable polymerase as previously described, and a buffer solution.

In a further aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for a specific mutation, the presence of which gives rise to fungicide resistance, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

In a further aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide comprising contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid, the presence of which gives rise to fungicide resistance, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended only when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

In a yet further aspect the invention provides a method of selecting an active fungicide and optimal application levels thereof for application to a crop comprising analysing a sample of a fungus capable of infecting said crop and detecting and/or quantifying the presence and/or absence of a mutation in a gene from said fungus wherein the presence of said mutation may give rise to fungicide resistance and then selecting an active fungicide and optimal application levels thereof.

In a preferred embodiment of this aspect of the invention the detection method comprises contacting a test sample comprising fungal [illegible] is present in the sample; and detecting the presence or absence of the said mutation by [illegible]

quantification is achieved by contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid the presence of which gives rise to fungicide resistance, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended only when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

In a still further aspect the invention provides a method of controlling fungal infection of a crop comprising applying a fungicide to the crop wherein said fungicide is selected according to the selection method of the invention described above.

In a yet further aspect the invention provides an assay for the detection of fungicidally active compounds comprising screening the compounds against strains of fungi which have been tested for the presence or absence of a mutation giving rise to fungal resistance according to the methods of the invention described herein and then determining fungicidal activity against said strains of fungi.

The methods of the invention described herein are especially suitable for use with plant pathogenic fungal strains where the presence of a mutation in a cytochrome *b* gene gives rise to resistance to a strobilurin analogue or a compound in the same cross resistance group.

By applying the methods of the invention described herein the appropriate rate of application of fungicides and/or the appropriate combination of fungicides to be applied to the crop may be determined.

The methods of the invention described herein are particularly suitable for monitoring fungal resistance to a strobilurin analogue or a compound in the same cross resistance group in crops such as cereals, fruit and vegetables such as canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion, vines and turf.

The fungal cytochrome *b* gene may be mitochondrially encoded and this is particularly preferred. The methods of the invention described herein are particularly sensitive at detecting low frequency of mutations in mitochondrially encoded genes, such as

the cytochrome *b* gene, making this an especially useful and commercially important way of screening plant pathogenic fungi for the onset of fungicidal resistance wherein the resistance is due to a mutation in a mitochondrially encoded gene.

The invention will now be illustrated with reference to the following non-limiting Examples and Figures in which :

Figure 1 shows: a diagrammatic representation of the Scorpion system™ detection system

Figure 2 shows: a graph illustrating the detection of a serial dilution of *P.viticola* mutant DNA within a background of wild type DNA using the C specific primer

Figure 3 shows: a picture showing an *E.graminis* mass population screen

Figure 4 shows: a picture showing an *E.graminis* single spore isolate screen

Figure 5a shows: a graph illustrating *E.graminis* total DNA amplified with the three primer pairs (specific G/C and control primers) (in duplicate)

Figure 5b shows: a graph illustrating the amplification of a sensitive *E.graminis* isolate with the three primer pairs (in duplicate)

Figure 6 shows: a graph illustrating the amplification of a resistant *E.graminis* isolate with the three primer pairs (in duplicate)

Figure 7 shows: a plan describing the preparation of *Rhynchosporium secalis* isolates for ARMS assay

Figure 8a shows: a graph illustrating a serial dilution of the wild type *R.secalis* plasmid amplified with the wild type specific primer pair

Figure 8b shows: a graph illustrating the highest concentration of the wild type and mutant *R.secalis* plasmids amplified with the wild type specific primer pair

Figure 9a shows: a graph illustrating a serial dilution of the mutant *R.secalis* plasmid amplified with the mutant specific primer pair

Figure 9b shows: a graph illustrating the highest concentration of the wild type and mutant *R.secalis* plasmids amplified with the mutant specific primer pair

Figures 11 a, b and c show: graphs illustrating the amplification of the *R.secalis* DNA and DNA samples

Figures 12 a and b show: graphs illustrating the amplification of the *R.secalis* R5 isolate in two dilutions with the three primer pairs (in duplicate)

Figures 13 a and b show: graphs illustrating the amplification of the *R.secalis* pooled isolates in two dilutions with the three primer pairs (in duplicate)

Figure 14a shows: a plan describing the preparation of *Pyrenophora teres* isolate K1916 for the ARMS assay

Figure 14b shows: a plan describing the preparation of *P.teres* isolates for the ARMS assay

Figure 15a and b show: graphs illustrating the amplification of the *Pteres* P13 and P15 isolates in two dilutions with the three primer pairs (in duplicate)

Figure 16a, b and c show: graphs illustrating the amplification of the *Pteres* pooled isolates in two dilutions with the three primer pairs (in duplicate) and the negative control

EXAMPLES:

In the first three Examples below the Scorpion system™ (Zeneca Diagnostics) was used as a product detection system. This detection system is described in full in PCT application number PCT/GB98/03521 filed in the name of Zeneca Limited on 25 November 1998 the teachings of which are incorporated herein by reference. This novel detection system uses a tailed primer and an integrated signalling system. The primer has a template binding region and a tail comprising a linker and a target binding region. In use the target binding region in the tail hybridises to complementary sequence in an extension product of the primer. This target specific hybridisation event is coupled to a signalling system wherein hybridisation leads to a detectable change (see Figure 1). The detection method of this system offers a number of significant advantages over other systems. Only a single primer/detector species is required. This provides both increased simplicity and enhanced specificity based on the ready availability of the target binding region for hybridisation with the primer extension product. The newly synthesised primer extension product is the target species so the output signal obtainable is directly related to amount of extended primer. It is not dependent on additional hybridisation events or enzymatic steps. Intra and Inter-strand competition for the probe site is limited so probe design becomes simplified. As the interaction is unimolecular, the signalling reaction is very rapid, permitting increased cycling rates which is a significant feature for primer design.

The Scorpion primers designed in the examples described below had the following modifications in common:

- A hexethylene glycerol (HEG) monomer as a blocking moiety that is sited between the template binding region of the primer and the tail region, which moiety prevents polymerase mediated chain copying of the tail region of the primer template.
- A FAM fluorescent molecule is added to the 5'end of the primer. FAM is one of the fluorescence molecules that can, for example, be readily detected by the 488nm laser of the ABI PRISM 7700
- MR is a non-fluorogenic quencher attached to a uracil

Other fluorescence molecules and quenching mechanisms are also suitable in scorpion primer design and would be suitable to use in this invention.

In the last Example, an intercalating dye was used as a detection mechanism instead of the Scorpion method.

EXAMPLE 1

In Example 1, we describe a study of the incidence of $G_{143}A$ in *Plasmopara viticola* field isolates.

The wild type (strobilurin analogue 1-sensitive ES2B) isolate of *Plasmopara viticola* (causal agent of vine downy mildew) was collected in 1996 from Spain. This isolate had never been exposed to strobilurin analogue selection. Infected vine leaves were hand picked and stored in a polythene bag and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival the leaves were placed in pairs, with abaxial sporulating surfaces together, over moist absorbent paper in plastic boxes, and incubated for 24-48 hours at 21-24°C. Single lesions were excised from the leaves and the sporangia suspended in 5mls deionised water, then sprayed to maximum retention onto the abaxial surfaces of 5-6 week old vine seedlings (Ohanez). Freshly inoculated plants were incubated for 24 hours in

infection showed as sporulating lesions on the abaxial leaf surfaces. Further subculturing

Partial *Plasmopara viticola* cytochrome *b* sequence was amplified using a panel of primers based on conserved regions between *Phytophthora megasperma* and *Aspergillus nidulans* sequences. DNA was extracted from the strobilurin-sensitive isolate, using a phenol/chloroform extraction protocol. Sporangia were washed into 30mls of double distilled H_2O (ddH_2O) from six leaves with 90-100% disease cover (originated from artificially inoculated six week old vine seedlings). The sporangial suspension was filtered through miracloth (Calbiochem cat # 475855) and centrifuged at 3600rpm for 10mins at 4°C. The sporangia were then frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar. 0.5mls of lysis buffer (200mM Tris-HCl pH8.5, 250mM NaCl, 25mM EDTA and 0.5% SDS) was added and the material was transferred to a sterile screw cap Eppendorf tube. 0.5mls of phenol/chloroform/isoamyl alcohol (25:24:1) mixture was immediately added and mixed by inverting several times. The Eppendorf tubes were centrifuged for 30 mins at 14000rpm and the aqueous phase transferred to fresh tubes. This phenol/chloroform/isoamyl alcohol extraction was repeated but this time the tubes were centrifuged at 14000rpm for only 15mins. After transferring the aqueous phase to a clean tube, a final chloroform extraction was performed. The DNA was precipitated by adding 0.1 vol of 3M NaAcetate and 0.6 vol of isopropanol. After inverting several times, the tubes were centrifuged at 14000rpm for 20mins at 4°C. The DNA was washed twice with 70% ethanol, vacuum dried and resuspended in 50µls of ddH_2O. The DNA yield was confirmed by gel electrophoresis and a serial dilution of the DNA (1:10, 1:100 and 1:1000) was made in ddH_2O for the use as template material in subsequent PCR reactions. PCR reactions were set up as recommended by the manufacturer of the Taq Polymerase enzyme (Gibco) and the primers were added to the reactions to a final concentration of 1pmole/µl . 10µls of each DNA dilution was added to the appropriate PCR reactions. Relevant procedures were carried out in order to limit the risk of PCR contamination. 30 cycles of 94°C for 45sec, 42°C for 45sec and 72°C for 1min30sec were carried out. A final extension at 72°C for 10mins was also performed. The efficiency of the PCR reactions was then assessed by analysing 18µls of the PCR reactions by gel electrophoresis. A 2µl sample of the PCR products was cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed in *E.coli* cells (as per the manufacturer's recommendations). A series of resulting clones were checked for the presence of inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis

using *EcoRI*. 6 clones with suitable inserts were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer). When the sequence data was analysed with the relevant bioinformatics software (Seqman, Editseq and Macaw software), the resulting novel sequence encoded for a new cytochrome *b* gene that had close homology to other Oomycete cytochrome *b* sequences known. The *Plasmopara viticola* specific primers that were used in later amplifications of the cytochrome *b* region of interest were PLAS17F 5' AAATAACGGTTGGTTAATTCG 3' and PLAS15R 5' TCTTAAAATTGCATAAAAAGG 3' (covering amino acid region 73-283 according to the *S.cerevisiae* numbering system).

A strobilurin-resistant isolate of *Plasmopara viticola* was found at one site during monitoring of field populations in 1998. Infected vine leaves were collected and processed as above but samples were subcultured as mass populations and not isolated as single lesions prior to testing. The test method was a 24 hour preventative spray onto 4-week old vine seedlings. A chemical dilution series was prepared by dissolving 5mgs strobilurin analogue 1 (technical material, 97% pure) in 1ml acetone and carrying out a further dilution with deionised water at room temperature to give a rate of 3ppm. The abaxial surfaces of the target leaves were sprayed using a DeVilbiss spray gun, 10psi, to maximum retention. Control plants were sprayed with deionised water only. The treated plants were left to dry in a growth room (conditions as above) overnight. Inoculation was carried out at 5,000 sporangia per ml and freshly inoculated plants incubated as described previously. 7 days following inoculation any putatively resistant growth on the treated leaves was isolated and subcultured to provide sufficient material for PCR analysis. This isolate was designated T5.

Partial cytochrome *b* gene sequence was amplified with PLAS17F and PLAS15R primers from the resistant isolate (T5). Total DNA material (genomic and mitochondrial) was extracted using the phenol/chloroform extraction protocol (as described previously). The DNA was then checked by gel electrophoresis and the DNA was diluted 1:10 and 1:100 in ddH_2O. 10µls of each DNA [illegible]

[illegible]

1pmole/µl. Relevant procedures were carried out to limit the risk of PCR contamination. 30 [illegible]

1min. A final extension at 72°C for 10mins was also carried out. The PCR reactions were performed in duplicate. After the analysis of 10µls of the PCR reactions by gel electrophoresis on an 0.8% TBE agarose gel, the resulting PCR products were pooled prior to cloning. A 1µl sample of the pooled PCR products was cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed in *E.coli* cells (as per the manufacturer's recommendations). A series of resulting clones were checked for the presence of inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 10 clones with suitable inserts were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer).

Analysis of the sequence data using suitable bioinformatics software (Seqman, Editseq and Macaw software) revealed a G-->C point mutation in the cytochrome *b* sequence in all 10 cases. This DNA point mutation would in turn lead to a Glycine to Alanine change at position 143 (according to the *S.cerevisiae* amino acid coding system). Different specific ARMS *P.viticola* primers were designed around this $G_{143}A$ point mutation:

Two forward ARMS primers based on the wild type sequence:

G-sp-f-1: CCTTGG**T**GACAAATGAGTTTTTG**T**GG

G-sp-f-2: CCTTGG**T**GACAAATGAGTTTTTGGAG

Two forward ARMS primers based on the $G_{143}A$ mutation:

C-sp-f-1: CCTTGG**T**GACAAATGAGTTTTTGG**CC**

C-sp-f-2: CCTTGG**T**GACAAATGAGTTTTTGGA**C**

A control primer designed upstream from the point mutation:

STAND: GCCTTGGGGACAAATGAGTTTTTG

*In all of the above ARMS primers, the -1 base (the 3'end base of the primer sequence) is the point mutation spot. The bases in bold differ from the wild type *Plasmopara viticola* cytochrome *b* sequence. In all of the ARMS primers (not the control primer), the -20 base was changed from a G to a T base. This was done to disrupt the run of G bases. In the G-sp-f-2 and C-sp-f-2 primers, the -2 position was changed from a G to a A base. In the G-sp-f-1, the -3 position was changed from a G to a T base. In the C-sp-f-1 primer, the -2 primer was changed from a G to a C base. These alterations to the sequence were made to destabilise the primer and render it more specific to the corresponding template. Examples in the literature

have shown that destabilising the ARMS primer decreases the risk of the primer mispriming off the wrong template (Newton et al. Nucleic Acid Research 17 (7) 2503-2516 1989).

The Scorpion product detection system™ was used in this case as a detection mechanism and the Scorpion reverse primer was

SCORPIO: FAM-CCCGCC**GTAATTGTAGGGGCTGTACTAATAC**GGCGGG MR-HEG-*GATACCTAATGGATTATTTGAACCTACCT*

* Underlined regions are the hairpin forming parts, FAM is the fluorescein dye, MR is a non-fluorogenic quencher attached to a uracil and HEG is the replication blocking hexethylene glycol monomer. The sequence in italics is the reverse primer sequence and the sequence in bold is the Scorpion sequence that binds to the extension product of the reverse primer.

All primers were synthesised by Oswel DNA service (Lab 5005, Medical and Biological Sciences building, Southampton). Before use, the primers were diluted to 5uM in a total volume of 500µls each. The primers were then further diluted to a final concentration of 500nM in the PCR reactions.

In all cases AmpliTaq Gold enzyme (Perkin-Elmer/ABI) was included in the reaction mix at 1units/25µls reaction. The reaction mix also contained 1xARMS buffer (10mM Tris-HCl, pH8.3, 50mM KCl, 1.2 mM $MgCl_2$, 0.01% gelatine), 100µM dNTPs and 1xROX (6-carboxy-rhodamine). Amplifications were performed in an ABI Prism 7700 machine for continuous fluorescence monitoring. A preliminary cycle of 94°C for 20 min was performed followed by 50 cycles of 94°C for 45sec and 60°C for 45sec. Fluorescence was monitored during the annealing/extension stage throughout the cycles.

The primers were first validated by using plasmid DNA as template at various concentrations. This was performed in order to check the specificity and sensitivity of the primer designs. Partial wild type cytochrome *b* sequence and the same sequence containing the $G_{143}A$ mutation were cloned in the TA Invitrogen pCR2.1 vector to be used in this validation process. The C-sp-f-2 and G-sp-f-2 primers was preferred to the C-sp-f-1 and the

[illegible] plasmid in a background of normal template was amplified using the ARMS C-sp-f-2 primer

detection is delayed. With each 10 fold dilution of the C plasmid, there is a delay of 4 cycles in the detection of fluorescence. It is encouraging to observe that when the C plasmid is present at only 1 in 10000 copies in wild type plasmid background, it is still detected by the specific ARMS primer. This primer is specific as no fluorescence can be detected in the 100% wild type plasmid sample in this experiment.

EXAMPLE 2

In Example 2, we report a study of the incidence of $G_{143}A$ in *Erysiphe graminis* f.sp. *tritici* field isolates.

Isolates of *Erysiphe graminis* f.sp. *tritici* and f.sp. *hordei* (causal agents of wheat and barley powdery mildew) were collected from Northern France, Germany, Ireland and the UK. This was done using two methods: hand collection of field leaves and air spora sampling by car-mounted jet spore trap (Burkhard Manufacturing Co. Ltd., Rickmansworth, UK).

Wheat leaves infected with sporulating powdery mildew were collected from sites where the populations had been exposed to strobilurin analogue 1 in previous and current field trials. Upon return to the Zeneca Agrochemicals Research centre at Jealott's Hill, the leaves were placed in polythene bags and incubated at 21°C overnight. The following day pustules were resporulating. All pustules were subcultured by tapping over fresh leaf pieces (wheat cv Rapier, 9 days old) placed over filter paper (Whatman No. 1) in 9cm petri dishes containing 1.2% tap water agar. The freshly inoculated plates were incubated for 7 days and then the resulting colonies were tested.

For spore trapping, wheat leaves were cut from 9 day old plants (cv. Rapier) and placed on 1.8% water agar in plastic dishes, and maintained at 5°C until required.
A jet spore trap was mounted on top of a car and the car was driven at speeds up to approximately 90 km/hr along prearranged routes in each country.
The plastic dishes containing the leaf pieces were placed in the base of the spore trap column where airborne spores entering the trap settled out onto the leaves. The dishes were changed approximately every 80 km. Once a batch of leaf pieces had been used in the spore trap, they were transferred to square petri dishes containing 60mls 1.8% water agar and filter paper and stored at 5°C.

On return to Jealott's Hill the leaves exposed in the spore trap were incubated in a constant temperature room (daylength 16hrs, light 4-5,000 lux, temperature constant 21°C, relative humidity ambient).

5-6 days after use in the spore trap pustules could be seen forming (small areas of yellowing of the leaf material following by appearance of powdery sporulating lesions). These were either subcultured onto leaf pieces in 9cm dishes as "mass populations" - one population per sampling stage, or excised as single pustule isolates and incubated separately on leaf pieces in 5cm petri dishes on 15mls 1.2% water agar covered with filter paper. The leaf pieces inoculated as mass populations were incubated for 7 days after which time sporulation was sufficient to inoculate a screen. Single spore isolates were incubated for 7 days but subcultured one further time to provide enough material for testing. Bulking up isolates if sporulation was poor was carried out by repeating the process as above until good (60-70%) sporulating disease coverage was obtained on all leaf pieces.

Wheat plants cv. Rapier were sown in plastic pots and incubated in a growth room (day 21°C/r.h. 60%; night 17°C/r.h. 95%; daylength 16 hours; 8,000 lux). On the 9th day following sowing the leaves were treated with chemical.

A chemical dilution series was prepared by dissolving 5mgs strobilurin analogue 1 (technical material, 97% pure) in 1ml acetone and carrying out a further dilution with a 0.05% Tween/deionised water solution at room temperature to give a rate of 5ppm. All leaf surfaces were sprayed using a DeVilbiss spray gun, 10psi, to maximum retention. Control plants were sprayed with a 0.05% Tween/deionised water solution only. The treated plants were left to dry in a growth room (conditions as above) overnight.

The following day 2.5 cm leaf pieces were excised from the treated plants and inserted into 1.8% TWA in 24.3cm x 24.3cm bioassay dishes as below. Plates were inoculated by settling tower using a compressed airline at approximately 4psi to blow spores off infected leaves from 9cm plates with 70-80% sporulating disease. The lids were replaced and the plates incubated in a 21°C constant temperature [illegible]

For the single spore isolate screen, test plants were prepared and treated as above. 24 hours post-treatment 2.5 cm leaf [illegible]

TWA in 5cm petri dishes. Leaf pieces were placed alternately, 4 per plate (2 at 0ppm, 2 at 5ppm). The leaf pieces were inoculated by tapping spores from sporulating leaves inoculated from single pustule lesions. 1 to 1 ½ leaf pieces (80% sporulating disease) were used to inoculate each 5cm plate. Plates were incubated at 21°C for 7 days.

After 7 days all isolates would be sporulating on the control (untreated) leaf pieces. Any growth on treated leaf pieces was considered putatively resistant. Material from these lesions was further subcultured onto strobilurin analogue 1-treated leaves to confirm resistance *in planta,* and analysed using the molecular assay described in this invention. Phenotypic resistance frequencies of approximately 1 in 100 and higher could be detected by the mass population screen (see Figure 3), and more precise frequencies were estimated by comparing single spore isolates where growth on treated leaves was comparable to controls (resistant), with plates where the treated leaves gave 100% control of disease (sensitive) (see Figure 4).

Partial *Erysiphe graminis* f.sp. *tritici* cytochrome *b* sequence was amplified using a panel of primers based on the conserved regions of *Aspergillus niger* and *Neurospora crassa* sequences. Approximately 500mgs of conidia from a strobilurin-sensitive isolate was tapped off leaves with sporulating disease, directly into 1.5ml Eppendorf tubes. DNA was extracted from this conidial sample from a strobilurin-sensitive isolate that has had no exposure to strobilurin analogue selection using a phenol/chloroform extraction protocol (see above). The resulting DNA was analysed by gel electrophoresis and a serial dilution of the DNA (1:10. 1:100 and 1:1000) was made in ddH_2O for the use as template material in PCR reactions. PCR reactions were set up as recommended by the manufacturer of the Taq Polymerase enzyme (Gibco) and the primers were added to the reactions to a final concentration of 1pmole/µl . 10µls of each DNA dilution was added to the appropriate PCR reactions. Relevant procedures were carried out in order to limit the risk of PCR contamination. 30 cycles of 94°C for 45sec, 42°C for 45sec and 72°C for 1min30sec were carried out. A final extension at 72°C for 10mins was also performed. The efficiency of the PCR reactions was then assessed by analysing 18µls of the PCR reactions by gel electrophoresis. A 2µl sample of the PCR products was cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed in *E.coli* cells (as per the manufacturer's recommendations). A series of resulting clones were checked for the presence of inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 6 clones with suitable

inserts were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer). When the sequence data was analysed using the relevant bioinformatics software, the resulting novel sequence encoded for a new cytochrome *b* gene that had close homology to other Ascomycete cytochrome *b* sequences known. The *Erysiphe graminis* specific primers that were used in later amplifications of the cytochrome *b* region of interest were ERY11F 5' ATGAACAATTGGTACAGTAAT 3' and ERY12R 5' GTTAGGTATAGATCTTAATAT 3' (that cover the amino acids region 114-287 according to the *S.cerevisiae* coding system)

Partial cytochrome *b* sequence was amplified with ERY11F and ERY12R primers from an initial strobilurin-resistant mass population isolate. Conidia material (~200mg) was resuspended in 200µls of ddH_2O and diluted 1:10, 1:100 and 1:1000 in ddH_2O. 10µls of each conidia dilution was added to Ready.to.go™ Taq polymerase PCR beads (Amersham Pharmacia Biotech product number 27-9555-01) and made up to 25µls with ERY11F and ERY12R primer solutions so that the final primer concentration was 1pmole/µl. Relevant procedures were carried out to limit the risk of PCR contamination. 30 cycles of a PCR reaction was carried out at 94°C for 45sec, 52°C for 45sec and 72°C for 1min30sec. A final extension at 72°C for 10mins was also carried out. The PCR reactions were performed in triplicate. After the analysis of 10µls of the PCR reactions by gel electrophoresis on an 0.8% TBE agarose gel, the resulting PCR products were pooled prior to cloning. A 2µl sample of the pooled PCR products was cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed in *E.coli* cells (as per the manufacturer's recommendations). A series of clones were checked for inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 10 clones with suitable inserts were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer).

Analysis of the sequence data using suitable bioinformatics software revealed a G-->C point mutation in the cytochrome *b* sequence in all 10 cases. This DNA point mutation in [illegible] sequence [illegible] that the G-->C mutation was indeed the cause for resistance to the strobilurin analogue compounds in *Erysiphe graminis* f.sp. *tritici*.

The cytochrome *b* sequence analysed from *Erysiphe graminis* f.sp. *hordei* was shown to be identical to the *Erysiphe graminis* f.sp. *tritici* sequence.

Different specific ARMS *E.graminis* primers were designed around this $G_{143}A$ point mutation:

A forward ARMS primer based on the wild type sequence:

G-sp-1: CCATACGGGCAGATGAGCCACTGGAG

A forward ARMS primer based on the $G_{143}A$ mutation:

C-sp-1: CCATACGGGCAGATGAGCCACTGG**AC**

A control primer designed upstream from the point mutation:

STAND2: GCCATACGGGCAGATGAGCCACTG

* In both the G-sp-1 and the C-sp-1 primers, the -1 base is the point mutation spot. The bases that differ from the wild type cytochrome *b E.graminis* sequence are in bold. The -2 position was changed from a G to a A base. This was done to destabilise the primer.

The Scorpion product detection system™ was used in this case as a detection mechanism and the Scorpion reverse primer was designed as recommended by the inventors :

SCORPIO 2: FAM-CCCGCC**GTTTTAGCTGCTTTAGCTTTAATGC**GGCGGG MR-HEG-*AACACCTAAAGGATTACCAGATCCTGCAC*

* Underlined regions are the hairpin forming parts, FAM is the fluorescein dye, MR is a non-fluorogenic quencher attached to a uracil and HEG is the replication blocking hexethlene glycol monomer. The sequence in italic is the reverse primer sequence and the sequence in bold is the Scorpion sequence that binds to the extension product of the reverse primer.

All primers were synthesised by Oswel DNA service. Before use, the primers were diluted to 5uM in a total volume of 500µls each. The primers were then further diluted to a final concentration of 500nM in the PCR reactions.

The primers were first validated by using plasmid DNA and total DNA as template at various concentrations. This was performed in order to check the specificity and sensitivity of the primer designs. Partial wild type cytochrome *b* sequence and the same sequence containing the $G_{143}A$ mutation were cloned in the TA Invitrogen pCR2.1 vector to be used in this validation process. The DNA material was extracted from a strobilurin-sensitive control

isolate using a phenol/chloroform extraction method (as described previously). As this is a "baseline" isolate that has never been subjected to chemical, this isolate represents an isolate prior to strobilurin analogue selection. Conidia samples from a French strobilurin-sensitive isolate (F12C) and a German strobilurin-resistant isolate(11-8) were then tested using the validated primers at two conidial dilutions.

In all cases AmpliTaq Gold enzyme (Perkin-Elmer/ABI) was included in the reaction mix at 1units/25µls reaction. The reaction mix also contained 1xARMS buffer (10mM Tris-HCl, pH8.3, 50mM KCl, 1.2 mM MgCl2, 0.01% gelatine), 100uM dNTPs and 1xROX. Amplifications were performed in an ABI Prism 7700 machine for continuous fluorescence monitoring. A preliminary cycle of 94°C for 20min was performed followed by 50 cycles of 94°C for 45sec and 60°C for 45sec. Fluorescence was monitored during the annealing/extension stage throughout the cycles.

When tested against the control templates, the primers showed adequate specificity with no evidence of mispriming occurring of the wrong template. In Figure 5a, the reactions of *E.graminis* DNA at a 1:100 dilution with the three primer mixes (Stand 2 + Scorpio 2, G-sp-1 + Scorpio 2 and C-sp-1 + Scorpio 2) are shown. Each reaction was carried out in duplicate. The control and G specific primer reactions emit a good fluorescence signal whilst the C specific primer reaction does not show any fluorescence being produced. The control and G-specific ARMS primers have recognised and bound to the template whilst the C-specific primer did not bind to the template present in the reaction. In this case, the genotype analysis is showing only the wild type genotype being present.

Figure 5b illustrates the PCR reactions where the French sensitive isolate (F12C) was amplified with the three primer mixes (Stand 2 + Scorpio 2, G-sp-1 + Scorpio 2 and C-sp-1 + Scorpio 2). In each case ~200mg of conidia material was resuspended in 200µls of ddH_2O and diluted 1:100 and 1:1000 in ddH_2O. 5µls of the dilutions were added to the appropriate PCR reactions. Here again, the control and G-specific primer reactions emit a good signal

[illegible] template for the reaction. This is either due to the reduced copies of molecules that can be

in the conidial sample. Either way, using a direct fungal sample in the reaction still gave a clear result.

Figure 6 illustrates the PCR reactions where the German resistant isolate at two dilutions was amplified using the three primer mixes (Stand 2 + Scorpio 2, G-sp-1 + Scorpio 2 and C-sp-1 + Scorpio 2). The control and C-specific primer reactions emit a good signal whilst the G-specific primer reaction does not show any fluorescence being produced. This indicates that only the mutant $G_{143}A$ genotype has been detected in this sample.

EXAMPLE 3

In Example 3, we report a study where various *Rynchosporium secalis* isolates were screened for the $G_{143}A$ mutation. This is an example where the $G_{143}A$ assay was carried out on a species where the point mutation had not yet been found.

The wild type isolates of *Rhynchosporium secalis* were collected from the UK and France during 1981 and 1998 (see Table 6: *Rhynchosporium secalis* isolate details). The 1981 isolate could be considered "baseline" (collected prior to use of strobilurins in the field) and 1998 isolates were obtained from Zeneca trial sites and had been exposed to several sprays of strobilurins over a number of seasons. Infected barley leaves were hand picked and stored in a polythene bag and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival at Jealott's Hill single lesions were excised from the leaves, surface sterilised in ethanol (30 seconds) followed by 0.1% sodium hypochlorite solution (2 minutes) then placed onto Lima Bean agar and incubated under alternating 12 hour black light/no light at a constant temperature of 19°C for 4-5 days. Colonies growing out of the lesions were subcultured by uncounted spore suspension onto Lima Bean agar and incubated as above for approximately 7 days until sporulation was obtained. Resulting spores were removed and stored in liquid nitrogen until isolates were required. Retrieval of isolates was achieved by plating out the spore suspension onto Lima Bean agar and incubating as above for approximately 7 days until sporulation was obtained.

Isolate code	Year collected	Country of origin
K1124	1981	UK
K3274	1998	UK

K3276	1998	UK
K3277	1998	UK
K3278	1998	UK
K3266	1998	UK

Table 6: *Rhynchosporium secalis* isolate details

Partial cytochrome *b* sequence was identified from two *R.secalis* isolates (K1124 and K3327). The isolates were grown from 100,000 spore per ml suspension in a medium with a non-fermentable carbon source shaking at 85rpm 21 days at 19°C (12hrs light/12hrs dark) and the mycelium was collected by filtering through a miracloth and frozen at -20°C until required. DNA was produced from the mycelia material by using a phenol/chloroform extraction protocol (see above). 2µls of the DNA was checked by gel electrophoresis and diluted (1:10, 1:100 and 1:1000) for use as template in PCR reactions. The PCR reactions were set up as described in earlier examples. The primers used were degenerate primers that were designed in the homologous regions of the cytochrome *b* fungal sequences (covering amino acid region 100 to 295 according to *S.cerevisiae* coding system) and *Erysiphe graminis* specific primers ERY11F and ERY12R. A band of the expected size was amplified using both primer pairs from each isolates and each PCR product was cloned in the Invitrogen pCR2.1 TA vector. Wizard minipreps were carried out to identify clones with suitable insert and 5 were submitted for sequencing from each using the M13 forward and reverse primers. Upon analysis with the relevant bioinformatics software, it was found that a novel cytochrome *b* sequence had been identified which was closely related to other Ascomycete cyt *b* sequences.

Different specific ARMS *R secalis* primers were designed around the $G_{143}A$ point mutation location:

Two forward ARMS primer based on the wild type sequence:

Two forward ARMS primer based on the $G_{143}A$ mutation location:

C-sp-2: CCTTATGGACAGATGTCTTTATGATC

A control primer designed upstream from the point mutation:

STAND3: TCCTTATGGACAGATGTCTTTATG

* In the ARMS primers, the -1 base (the 3`end base) is the point mutation spot. The bases that differ from the wild type cytochrome *b R.secalis* sequence are in bold. The -2 position was changed from a G to an A or T base. This was done to destabilise the primer.

The Scorpion product detection system™ was used in this case as a detection mechanism and the Scorpion reverse primer was designed as recommended by the inventors:

SCORPIO 3: FAM-CCCGCC**ATATTAGCTGCATTAGTATTAATGC**GGCGGG-MR-HEG-*TACACCTAAAGGATTACCTGACCCTGCAC*

* See previous examples for details.

All primers were synthesised by Oswel DNA service. Before use, the primers were diluted to 5uM in a total volume of 500µls each. The primers were then further diluted to a final concentration of 500nM in the PCR reactions.

The primers were first validated by using plasmid DNA as template at various concentrations. This was performed in order to check the specificity and sensitivity of the primer designs. Partial wild type cytochrome *b* sequence and a sequence containing the $G_{143}A$ mutation were cloned in the TA Invitrogen pCR2.1 vector to be used in this validation process. As this mutation has not been found in this isolate, the point mutation was incorporated into the sequence using a PCR strategy: the point mutation was incorporated into a primer design and was used to amplify the region of interest using the wild type clone as template. The PCR reactions were set up using standard methods as previously described and 30 cycles of 94°C for 45sec, 56°C for 45sec and 72°C for 1min30 were performed. A final extension time of 10mins at 72°C was also carried out. The resulting PCR product was cloned into the TA Invitrogen pCR2.1 vector and a resulting clone was sequenced to check for any PCR induced errors prior to use in this experiment.

Undiluted, the plasmids were calculated to be at around $2x10^{11}$ molecules per µl. The two plasmids were diluted to $2x10^7$, 10^5, 10^3 and 10^1 molecules/µl and 5µls were used of each dilution meaning that there was ~ $1x10^3$, 10^6, 10^4 and 10^2 molecules of plasmid in the respective PCR reactions. In figure 8a, which shows a serial dilution of the G plasmid detected with the G primer mix, the detection of fluorescence is delayed by ~ 4 cycles with

each 10 fold plasmid dilution. Figure 8b, which shows the highest concentration G (wt) and C (mutant) cassette amplified with the G-sp-2 primer mix, demonstrates that the primer has a good window of specificity. The G primer does not misprime off the C template until very late in the cycles even though the DNA template concentration is high (~10^8 molecules of template in reaction). The C-sp-2 primer set also show good specificity through the specific and non specific plasmid dilutions (figure 9a and 9b). G-sp-2 and C-sp-2 primer mixes were used in following experiments instead of G-sp-3 and C-sp-3 primer mixes.

The second part of this experiment was to compare using total DNA and cDNA as template for the PCR. Total DNA material was prepared from various isolates using a phenol-chloroform extraction method (as described above). Total RNA was extracted from 100mg of ground mycelia using the RN easy kit from Qiagen (according to the manufacturer's recommendation). First strand cDNA synthesis was then prepared from 1µg of total RNA using RT PCR with the Advantage RT-PCR Clontech kit (according to the manufacturer's recommendation). Total DNA and cDNA from three isolates (R3-K3278 of 0.1ppm, R6-K3274 of 0.1ppm and R9-K3276 of 0.1ppm - see below for details) were pooled. The total DNA pool was used as template diluted 1:100, 1:1000 and 1:10000 and the cDNA was used as template neat and diluted 1:5 and 1:10. In each case, 5µls of template was added to the PCR reactions. PCR conditions described in example 1 and 2 were also used in this case apart from 40 cycles of the PCR reaction were performed in this case instead of 50. Figure 10a, b and c illustrate total DNA and cDNA templates at three dilutions (dilution 1: total DNA (1:100) and cDNA (neat); dilution 2: total DNA (1:1000) and cDNA (1:5); dilution 3: total DNA (1:10000) and cDNA (1:10)) amplified using the G primer mix. Figures 11a, b and c illustrate the total DNA and cDNA templates amplified using the C primer mix. Fluorescence could be detected slightly earlier in the total DNA samples so in order to give us the best chance of detecting any C mutation, total DNA material at a dilution of 1:10 and 1:1000 was used in the third part of this experiment. It is interesting to point out that in the pooled isolates off 0.1ppm [illegible]

presence of $G_{143}A$ mutation. The isolates tested in this examples were prepared as described

Isolates were passed through varying concentrations of strobilurin analogue 2 in a medium with a non-fermentable carbon source to obtain material for ARMS diagnosis. (See Figure 7: Preparation of *Rhynchosporium secalis* isolates for ARMS sequencing.) An initial spore suspension was inoculated (1ml at 100,000 spores/ml) into conical flasks containing chemically amended broth (1 flask per isolate per concentration). The material was incubated at 85rpm on an orbital shaker under 12 hours white light/12 hours no light at a constant temperature of 19°C. After growth was visible and there was sufficient material, mycelium was either submitted for ARMS diagnosis or further subcultured at an increased rate of strobilurin analogue 2 (see Figure 7) before testing.

In each of the following cases (see Table 7), DNA was prepared using a phenol/chloroform extraction protocol (see above) and checked by gel electrophoresis. A serial dilution of the DNA was made (1:10 and 1:1000) and in each case 5µls was added to the PCR reactions. In this case, 50 PCR cycles were carried out using PCR conditions described in previous examples.

isolate#	Plant Path name	culture conditions
isolate R1	K3278	0ppm
isolate R2	K3278	0.01ppm
isolate R3 - pool	K3278	0.1ppm
isolate R4	K1124	0ppm (pre Selection)
isolate R5	K3274	0ppm
isolate R6 - pool	K3274	0.1ppm
isolate R7	K3277	0.01ppm
isolate R8	K3266	0.01ppm
isolate R9 - pool	K3276	0.1ppm

Table 7: *Rhynchosporium secalis* isolate details

Data was generated for 6 individual isolates (R1, R2, R4, R5, R7 and R8) and 1 pool of isolates off 0.1ppm selection. Figures 12 a and b and figures 13 a and b show a single isolate grown off selection (R5 = K3274 off 0ppm) and the pooled isolates off 0.1ppm selection (K3278, K3274 and K3276) respectively with the three primer pairs in duplicate. In a figures, the graphs show the DNA template at 1:10 dilution whilst in the b figures, the graphs show the DNA template at 1:1000 dilution.

There is no evidence from these experiments to suggest that the C mutation is present at any significant level in any of these isolates. If the fluorescence detected with the C primer mix at cycle 40 was due to the presence of low frequency point mutations, it is still 20 cycles after fluorescence is detected with the G primer indicating that if the C mutation, if at all present, is at a frequency of no more than 1 in a million. This late detection in fluorescence is most likely however due to the primer mispriming.

EXAMPLE 4

In Example 4, we report a study where the $G_{143}A$ assay was carried out in *Pyrenophora teres* isolates. Again this is a species where the $G_{143}A$ mutation has not yet been identified.

The wild type isolates of *Pyrenophora teres* (causal agent of barley net blotch) were collected from the UK and France during 1994, 1996 and 1998 (see Table 8: *Pyrenophora teres* isolate details). 1994 and 1996 isolates could be considered "baseline" (collected prior to use of strobilurins in the field) and 1998 isolates were obtained from Zeneca trial sites and had been exposed to several sprays of strobilurins over a number of seasons. Infected barley leaves were hand picked and stored in a polythene bag and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival at Jealott's Hill the leaves were placed over moist absorbent cotton wool plastic boxes, and incubated for 24-48 hours at 21°C. Single lesions were excised from the leaves, surface sterilised in ethanol (30 seconds) followed by 0.1% sodium hypochlorite solution (2 minutes) then placed onto Rose Bengal agar and incubated under alternating 12 hour black light/no light at a constant temperature of 22°C for 4-5 days. Colonies growing out of the lesions were subcultured by mycelial plug onto V8+streptomycin agar and incubated as above.

Resulting mycelial material was removed and stored in liquid nitrogen until isolates were required. Retrieval of isolates was achieved by plating out mycelial material onto V8

Isolate code	Year collected	Country of origin
K1056	1980	Ireland
K1916	1994	UK
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

K2385	1996	UK
K2390	1996	UK
K2396	1996	UK
K3230	1998	UK
K3237	1998	UK
K3238	1998	UK
K3253	1998	UK

Table 8: *Pyrenophora teres* isolate details

Partial cytochrome *b* sequence was identified from two isolates (K1056 and K1916). The initial wild type sequence was obtained from material prepared by inoculating potato dextrose broth with a macerated mycelial suspension. The flask was incubated at 85rpm on an orbital shaker under 12 hours white light/12 hours no light at a constant temperature of 19°C for 21 days until there was sufficient mycelial material for the DNA extraction protocol. The mycelium was harvested by filtering through miracloth and was frozen at -20°C until needed. Total DNA was produced using the phenol/chloroform extraction protocol. After checking the DNA by gel electrophoresis, the DNA was diluted (1:10, 1:100 and 1:1000) and used as template in PCR reactions. Many different primer combinations were tried (specific and degenerates) and in most cases this was done to no avail. A primer pair that was designed to the *Aspergillus niger* and *Neurospora crassa* cytochrome *b* sequences did however give a PCR product. This product was cloned in the TA Invitrogen pCR2.1 vector and 6 clones were sequenced using M13 forward and reverse primers. Upon analysis of the sequencing data, we discovered that the reverse primer had correctly bound to cytochrome *b* sequence whilst the forward primer misprimed in what looked like intron sequence further upstream in the sequence. A specific *P.teres* primer was designed in the novel stretch of cytochrome *b* gene and was used with a forward primer that was designed based on *Venturia inaequalis* cytochrome *b* sequence on *P.teres* cDNA template. cDNA was produced from mycelium for the two isolates. Total RNA was extracted from 100mg of ground mycelium using the RN easy kit from Qiagen (according to the manufacturer's recommendation). First strand cDNA synthesis was prepared from 1ug of total RNA using RT PCR with the Advantage RT-PCR Clontech kit (according to the manufacturer's recommendation). 5µls of the resulting cDNA was then used in PCR reactions. A PCR product was amplified for both isolates (covering amino acid region 48 to 311 according to

the *S.cerevisiae* coding system). In both cases, it was cloned in the TA pCR2.1 Invitrogen vector and 4 positive clones were sequenced using M13 forward and reverse primers for each isolate. Sequence data analysis revealed that a novel cytochrome *b* sequence had been isolated that was closely related to other Ascomycete cytochrome *b* sequences.

Specific ARMS *P.teres* primers were designed around the $G_{143}A$ point mutation location:

A forward ARMS primer based on the wild type sequence:

G-sp-4: CCCTACGGGCAAATGAGCCTTTGAAG

A forward ARMS primer based on the $G_{143}A$ mutation location:

C-sp-5: CCCTACGGGCAAATGAGCCTTTGA**TC**

A control primer designed upstream from the point mutation:

STAND4: ACCCTACGGGCAAATGAGCCTTTG

The reverse primer used was:

UNLS4: TACACCTAAAGGATTTCCTGACCCTGCAA

* In the ARMS primers, the -1 base (the 3'end base) is the point mutation spot. The bases that differ from the wild type cytochrome *b P.teres* sequence are in bold. The -2 position was changed from a G to an A or T base. This was done to destabilise the primer.

All primers were synthesised by Oswel DNA service. Before use, the primers were diluted to 5uM in a total volume of 500µls each. The primers were then further diluted to a final concentration of 500nM in the PCR reactions.

In this case, we made use of an intercalating dye for the detection of PCR product. The dye used was YO-PRO-1 dye (Molecular Probes, Seattle Washington, USA) which binds to double stranded DNA and emits fluorescence which is detected by the ABI7700.

The primers were first validated by using plasmid DNA as template at various concentrations. This was performed in order to check the specificity and sensitivity of the

[illegible] and in this isolate, the point mutation was incorporated into the sequence using a PCR strategy: the point mutation was incorporated

as template. The PCR reactions were set up using standard methods as previously described and 30 cycles of 94°C for 45sec, 56°C for 45sec and 72°C for 1min30 were performed. A final extension time of 10mins at 72°C was also carried out. The resulting PCR product was cloned into the TA Invitrogen pCR2.1 vector and a resulting clone was cloned to check for any PCR induced errors prior to use in this experiment.

Undiluted, the plasmids were calculated to be at around $2x10^{11}$ molecules per µl. The two plasmids were diluted to $2x10^{7}$, 10^{5}, 10^{3} and 10^{1} molecules/µl and 5µls were used of each dilution meaning that there was ~ $1x10^{8}$, 10^{6}, 10^{4} and 10^{2} molecules of plasmid in the respective PCR reactions. The PCR conditions used were as described above where 50 cycles were carried out. The only difference was that YO-PRO-1 dye was added to the reaction mix. Graphs relating to this experiment are not shown but both specific primers showed good specificity. The generation of primer dimer product interfered with the signal past cycle 35. This method of detection is less sensitive than Scorpions because it is more affected by background noise and the dye recognises primer dimer products as well as PCR products. That said it was thought that valuable information could be drawn from using this method but increased caution has to be taken in interpreting the data.

In the following experiment, various different isolates were checked for the presence of $G_{143}A$ mutation. The isolates tested in this examples were prepared as described here:

Isolates were passed through varying concentrations of strobilurin analogue 2 in a medium with a non-fermentable carbon source to obtain material for ARMS diagnosis. (See Figure 14a: Preparation of *Pyrenophora teres* isolate K1916 for the ARMS assay and Figure 14b: Preparation of *Pyrenophora teres* isolates for the ARMS assay.) An initial spore suspension, where obtained, was inoculated (1ml at 100,000 spores/ml) into conical flasks containing amended broth (1 flask per isolate per concentration). The material was incubated at 85rpm on an orbital shaker under 12 hours white light/12 hours no light at a constant temperature of 19°C. After growth was visible and there was sufficient material, mycelium was either submitted for ARMS diagnosis or further subcultured at increased rates of strobilurin analogue 2 (see Figures 14a & b) before testing.

cDNA material was prepared (as described previously) from each of the isolates described above. This was done to avoid designing primers within the complex intron/exon

organisation of the *P.teres* cytochrome *b* sequence. Some of the isolates were pooled as described below:

Isolate#	Isolate name	growth conditions
P1	K1916	0.04ppm
P2	K1916	0.16ppm
P3 - pool 1	K3238	0.01ppm
P4 - pool 1	K2346	0.01ppm
P5 - pool 1	K2396	0.01ppm
P6 - pool 1	K2390	0.01ppm
P7 - pool 2	K3230	0.02ppm
P8 - pool 2	K3237	0.02ppm
P9 - pool 3	K2383	0.01ppm
P10	K1916	0ppm
P11 - pool 3	K3253	0.01ppm
P12 - pool 3	K2385	0.01ppm
P13	K1916	0ppm
P14	K1916	30ppm
P15	K1916	100ppm

Table 9: *Pyrenophora teres* isolate details

All PCR reactions were set up as previously described and 50 cycles were carried out. The only difference was that YO-PRO-1 dye was added to the reaction mix. The three primer pairs were used on the three pools and the 6 single isolates tested. cDNA neat and diluted 1:10 were used as template, in all cases 5µls of template was added to the PCR reactions. Figures 15 a and b illustrate the P13 and P15 isolates in two dilutions, in duplicate with the three primer pairs. This experiment shows that even though P15 was grown in 100ppm selection, genotypically, it does not look any different to P13 which was grown without selection. Figures 16 a, b and c show pool 1 and 2 in two dilutions, in duplicate, with the three primer pairs and the negative control which contained no template DNA. The rest of the data is not shown but in those cases the C primer mix did not show any fluorescence until

... with conclusions were made.

- Even though the C primer mix is not much affected by the formation of primer dimer in the negative control, it was concluded that any fluorescence detected past cycle 35 was probably due to primer dimer formation. This is the case for P1,P2, P10, P14 and pool 3.
- Pool 1 and pool 2 are showing an interesting result. Fluorescence is detected with the primer C mix in both cases at cycle 30 and is thus ahead from fluorescence which is normally detected due to primer dimer formation. This might be due to a low level frequency of C mutation being present. There is a window of 10 cycles between fluorescence detected with the G and C primer indicating that the C point mutation if present is at a frequency of 1 in 1000.

CLAIMS

1. A method for detecting a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

2. A method according to claim 1 wherein the mutation is present in the cytochrome *b* gene.

3. A method according to claim 1 for the detection of a $G_{143}A$ mutation in a fungal cytochrome *b* gene, which method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the $G_{143}A$ mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when a $G_{143}A$ mutation is present in the sample; and detecting the presence or absence of the said $G_{143}A$ mutation by reference to the presence or absence of a diagnostic primer extension product.

4. A method according to any of the preceding claims wherein the fungal gene is present in a plant pathogenic fungus selected from the group comprising *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum,*

[illegible] cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds

of said nucleotide gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group.

6. A diagnostic primer according to claim 5 wherein the penultimate nucleotides (-2) or (-3) of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

7. One or more diagnostic primers for detecting a $G_{143}A$ mutation in a fungal cytochrome *b* gene selected from the group comprising

5'CCTTGGTGACAAATGAGTTTTTGGAC3'

5'CCATACGGGCAGATGAGCCACTGGAC3'

5'CCTTATGGACAGATGTCTTTATGATC3'

5'CCCTACGGGCAAATGAGCCTTTGATC3'

and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given above and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer.

8. A diagnostic kit comprising one or more of the diagnostic primers as claimed in claims 5 to 7, nucleotide triphosphates, polymerase, and buffer solution.

9. A method of detecting plant pathogenic fungal resistance to a fungicide comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for a specific mutation the presence of which gives rise to fungicide resistance in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

10. A method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide comprising contacting a test sample comprising

fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid the presence of which gives rise to fungicide resistance, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended only when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

11. A method according to claim 9 or claim10 wherein said fungicide is a strobilurin analogue or any other compound in the same cross resistance group.

12. A method according to claim 10 or 11 wherein the method of detection and quantifying is based on fluorescence detection of diagnostic primer extension products.

13. A method of selecting an active fungicide and optimal application levels thereof for application to a crop comprising analysing a sample of a fungus capable of infecting said crop and detecting and/or quantifying the presence and/or absence of a mutation in a gene from said fungus wherein the presence of said mutation may give rise to fungicide resistance and then selecting an active fungicide and optimal application levels thereof.

14. A method according to claim 13 wherein the detection method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

[illegible] wherein said fungicide is selected according to claim [illegible] claim [illegible]

16. A method according to claims 13 to 15 wherein the fungicide is a strobilurin analogue or any other compound in the same cross resistance group.

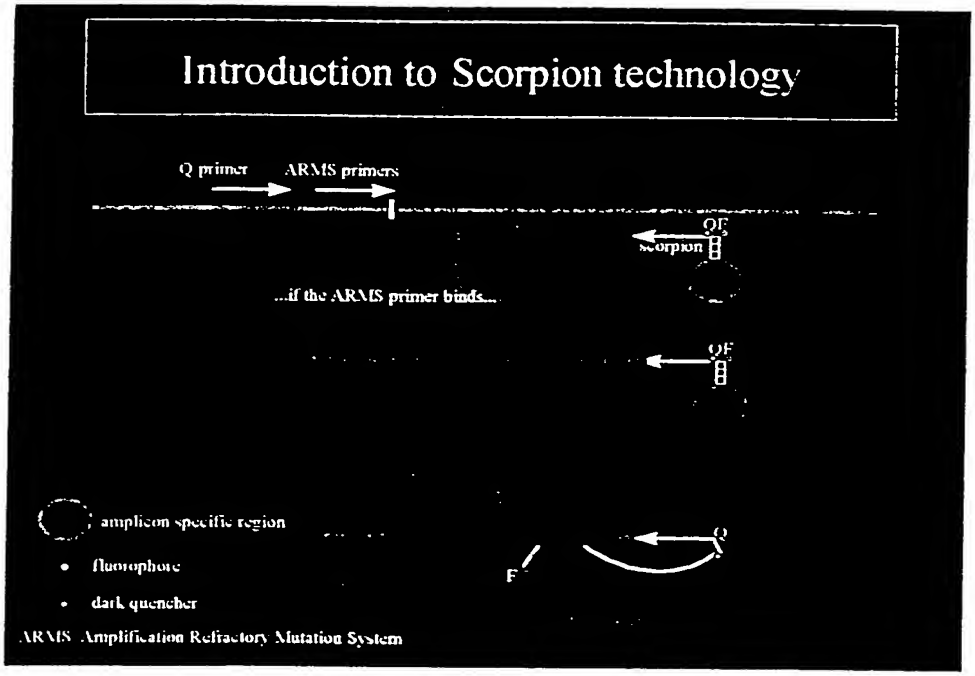

Introduction to Scorpion technology
Q primer
ARMS primers
Scorpion
...if the ARMS primer binds...
amplicon specific region
fluorophore
dark quencher
ARMS Amplification Refractory Mutation System

Figure 1

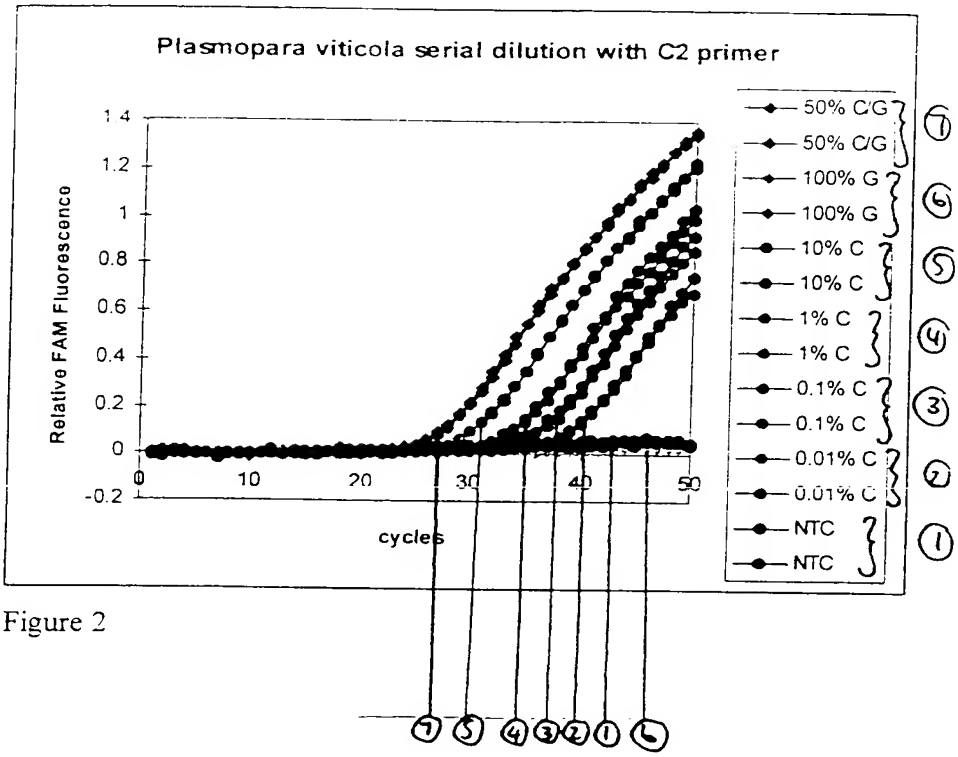

Plasmopara viticola serial dilution with C2 primer
Relative FAM Fluorescence
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
10
20
30
40
50
cycles
50% C/G
50% C/G
100% G
100% G
10% C
10% C
1% C
1% C
0.1% C
0.1% C
0.01% C
0.01% C
NTC
NTC
7
6
5
4
3
2
1

Figure 2

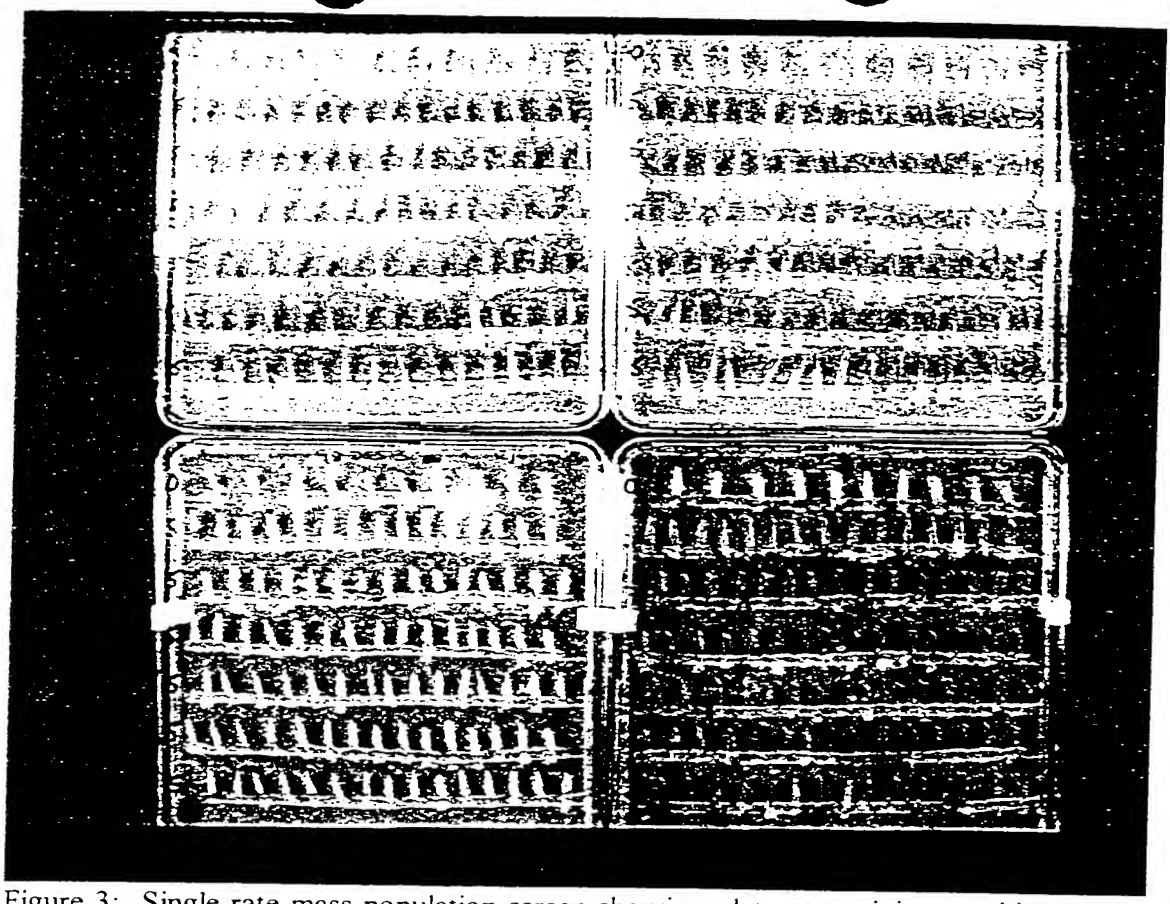


Figure 3: Single rate mass population screen showing plates containing sensitive and resistant isolates

Figure 4: Single spore resistance frequency detection assay

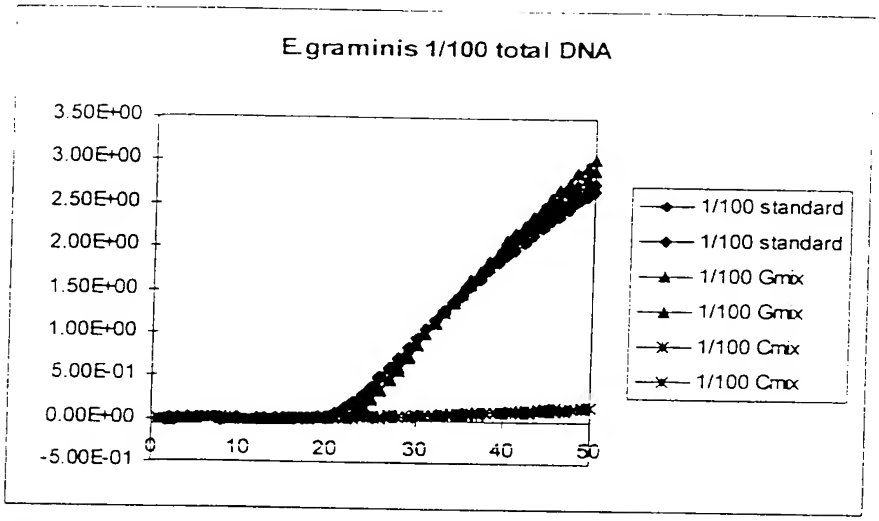


Figure 5a

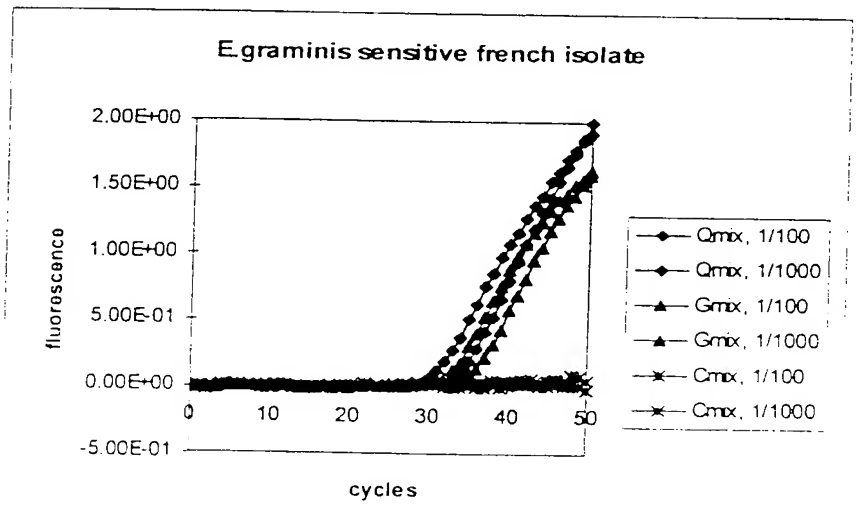


Figure 5b

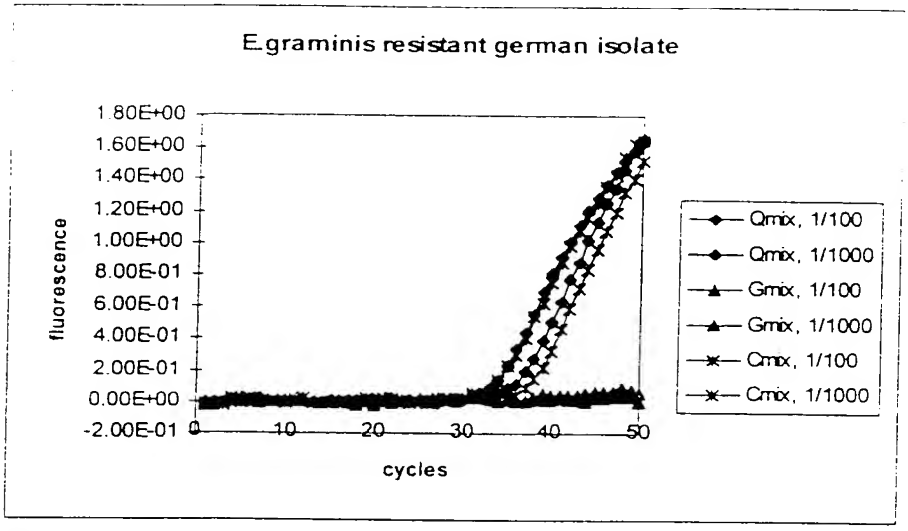

E.graminis resistant german isolate
1.80E+00
1.60E+00
1.40E+00
1.20E+00
1.00E+00
8.00E-01
6.00E-01
4.00E-01
2.00E-01
0.00E+00
-2.00E-01
fluorescence
0
10
20
30
40
50
cycles
Qmix, 1/100
Qmix, 1/1000
Gmix, 1/100
Gmix, 1/1000
Cmix, 1/100
Cmix, 1/1000

Figure 6

Preparation of *Rhynchosporium secalis* isolates for ARMS assay
(Selection in strobilurin analogue2)

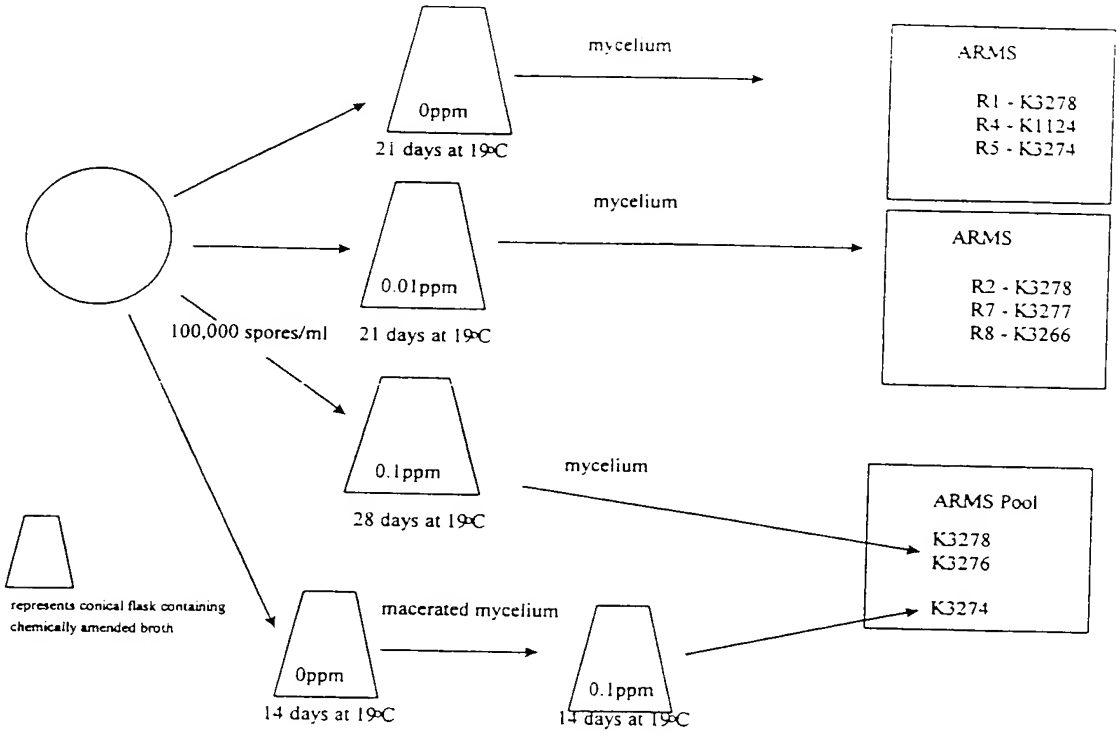


Figure 7

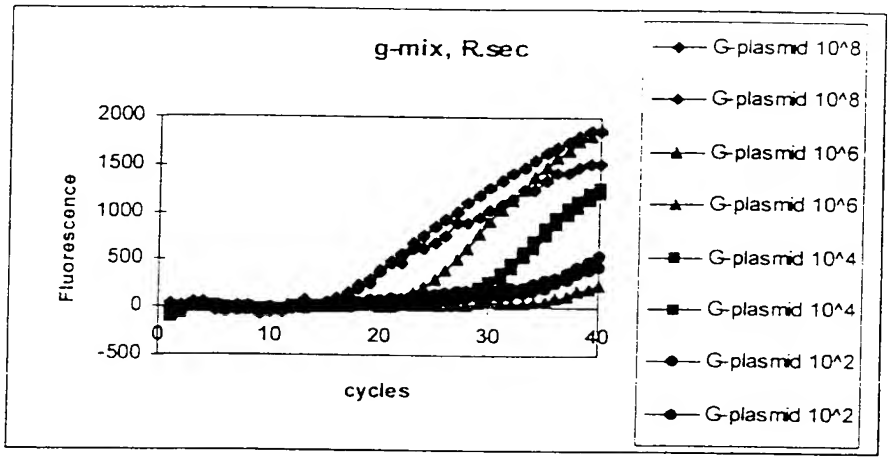


Figure 8a

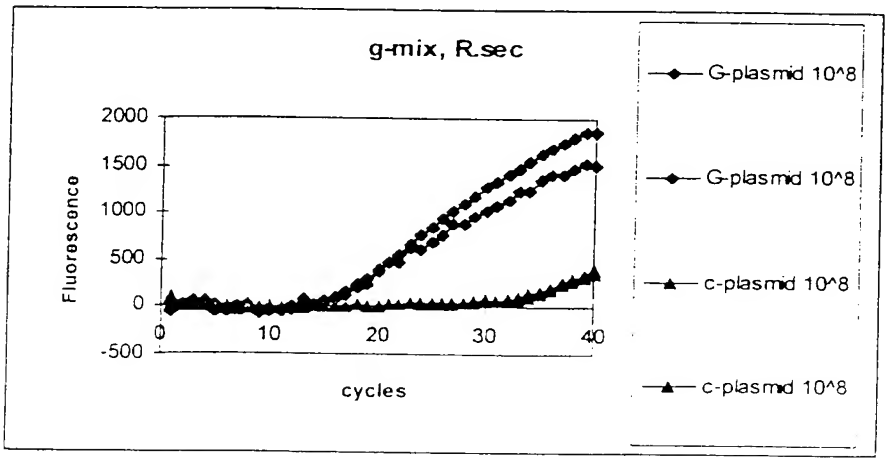


Figure 8b

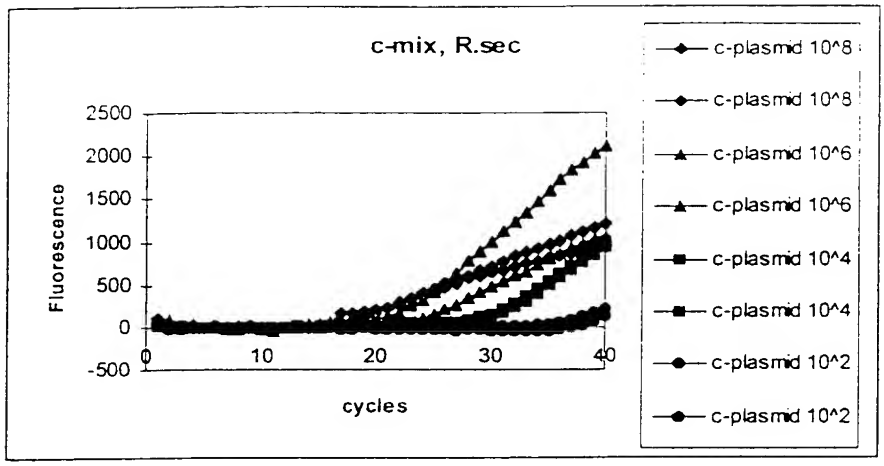


Figure 9a

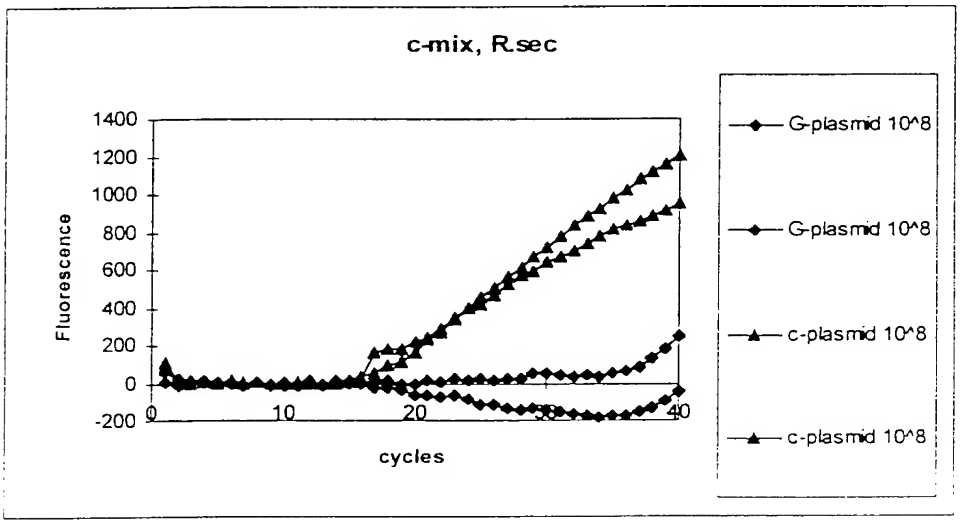
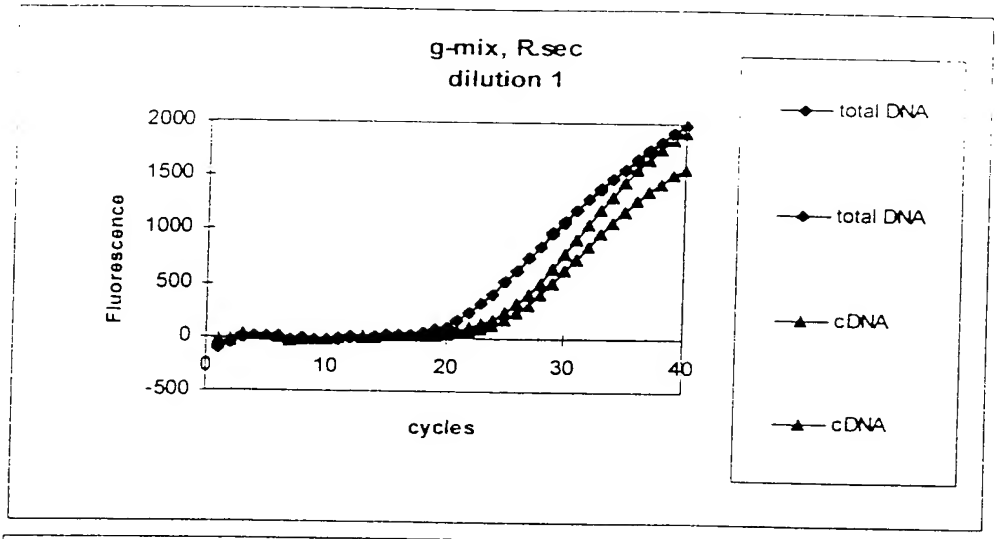
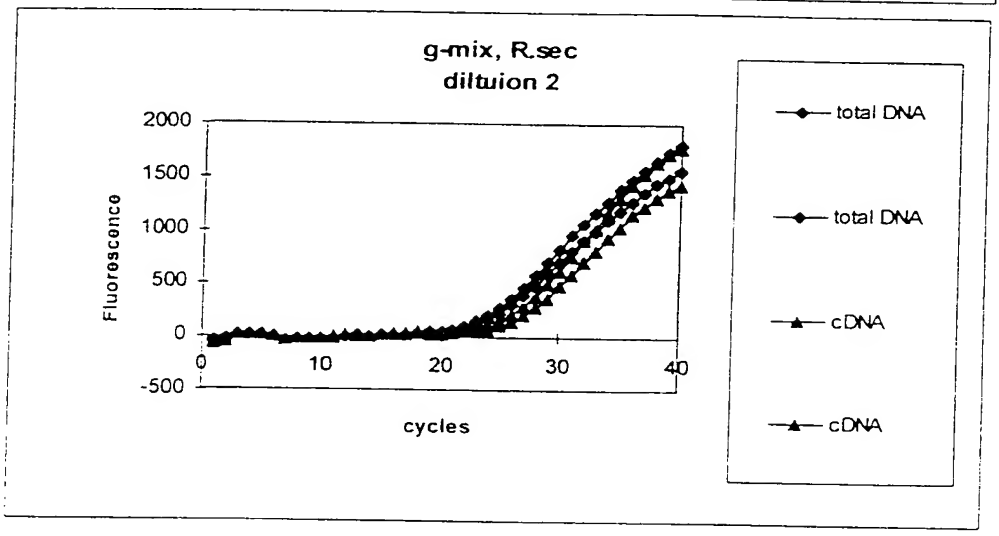
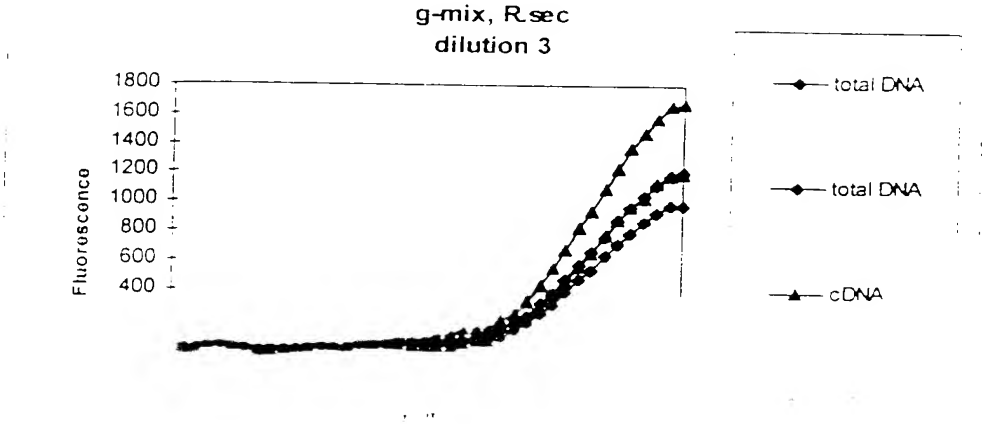
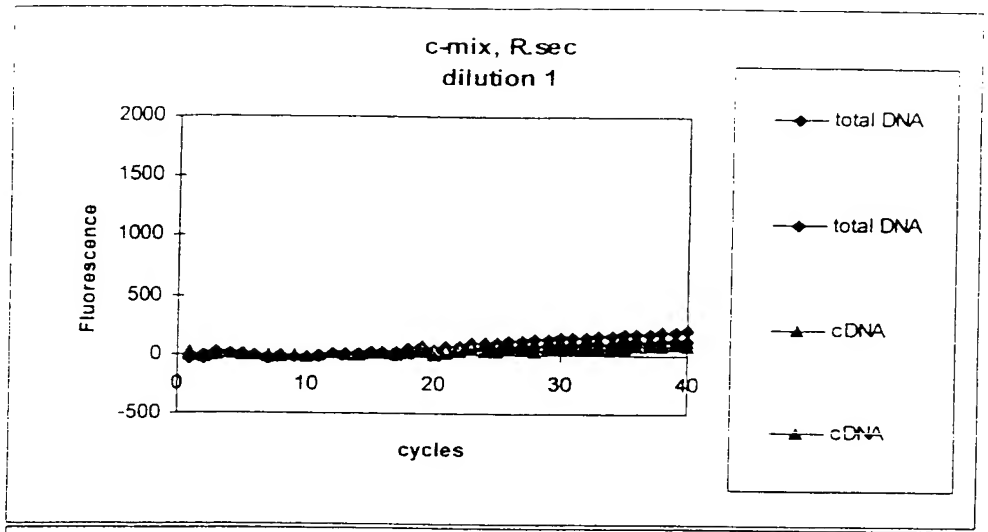


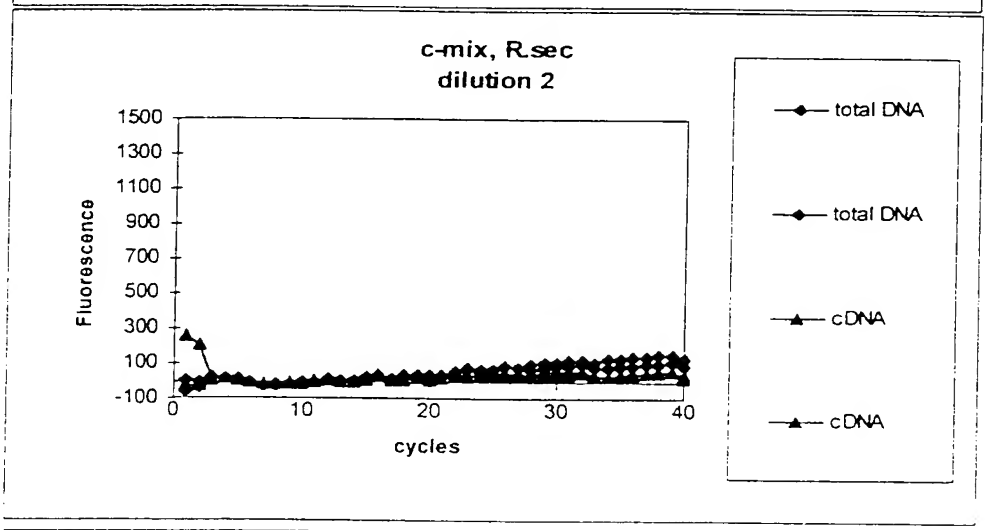
Figure 9b

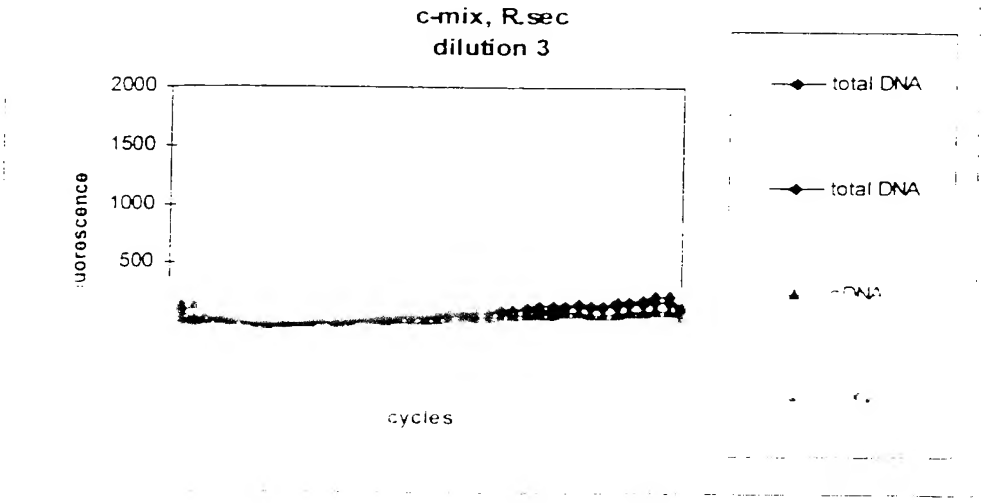

g-mix, R.sec
dilution 1
Fluorescence
2000
1500
1000
500
0
-500
0
10
20
30
40
cycles
total DNA
total DNA
cDNA
cDNA


g-mix, R.sec
diltuion 2
Fluorescence
2000
1500
1000
500
0
-500
0
10
20
30
40
cycles
total DNA
total DNA
cDNA
cDNA


g-mix, R.sec
dilution 3
Fluorescence
1800
1600
1400
1200
1000
800
600
400
total DNA
total DNA
cDNA


c-mix, R.sec
dilution 1
Fluorescence
2000
1500
1000
500
0
-500
0
10
20
30
40
cycles
total DNA
total DNA
cDNA
cDNA


c-mix, R.sec
dilution 2
Fluorescence
1500
1300
1100
900
700
500
300
100
-100
0
10
20
30
40
cycles
total DNA
total DNA
cDNA
cDNA


c-mix, R.sec
dilution 3
2000
1500
1000
500
cycles
total DNA
total DNA

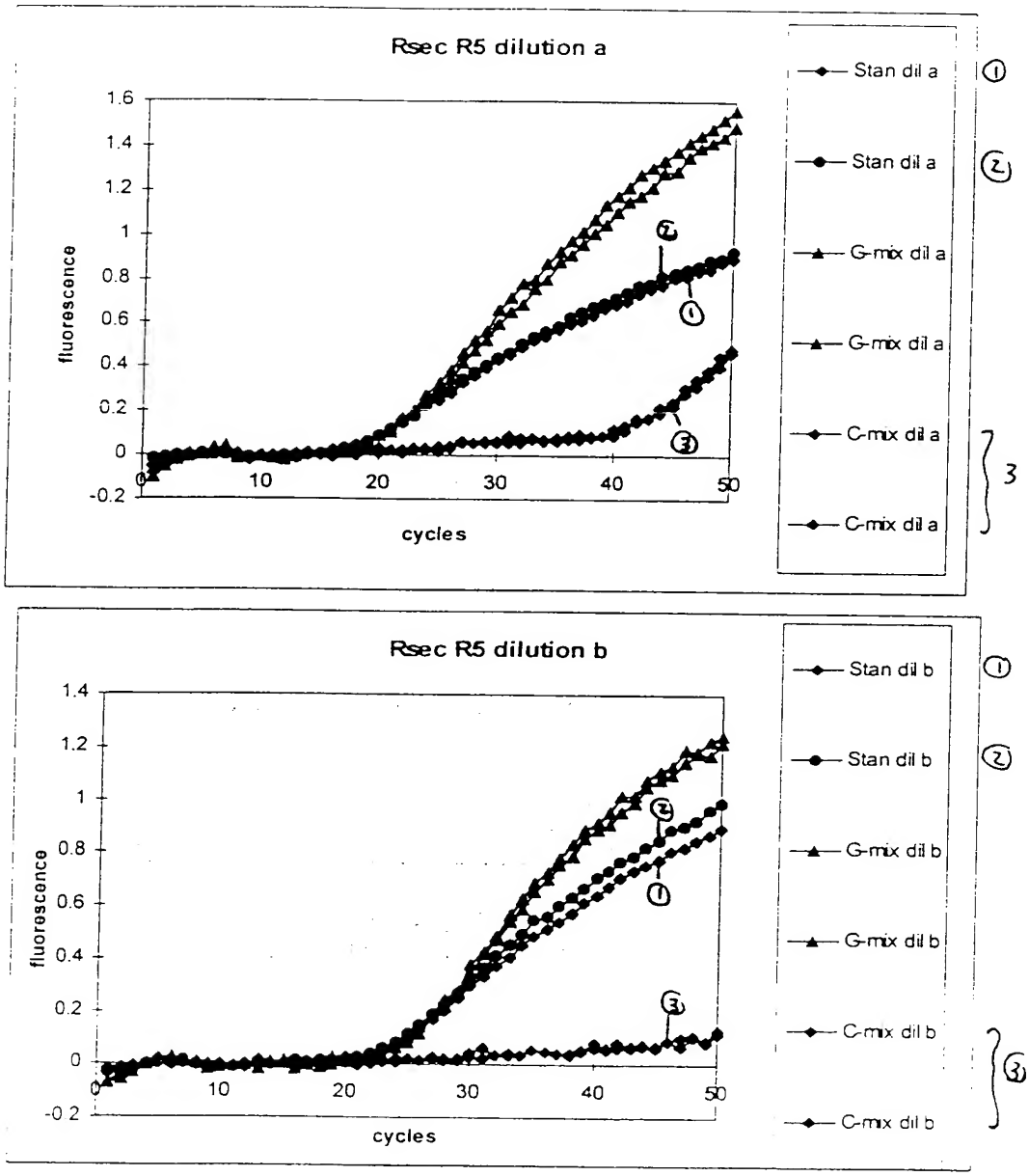


Figure 12 a and b

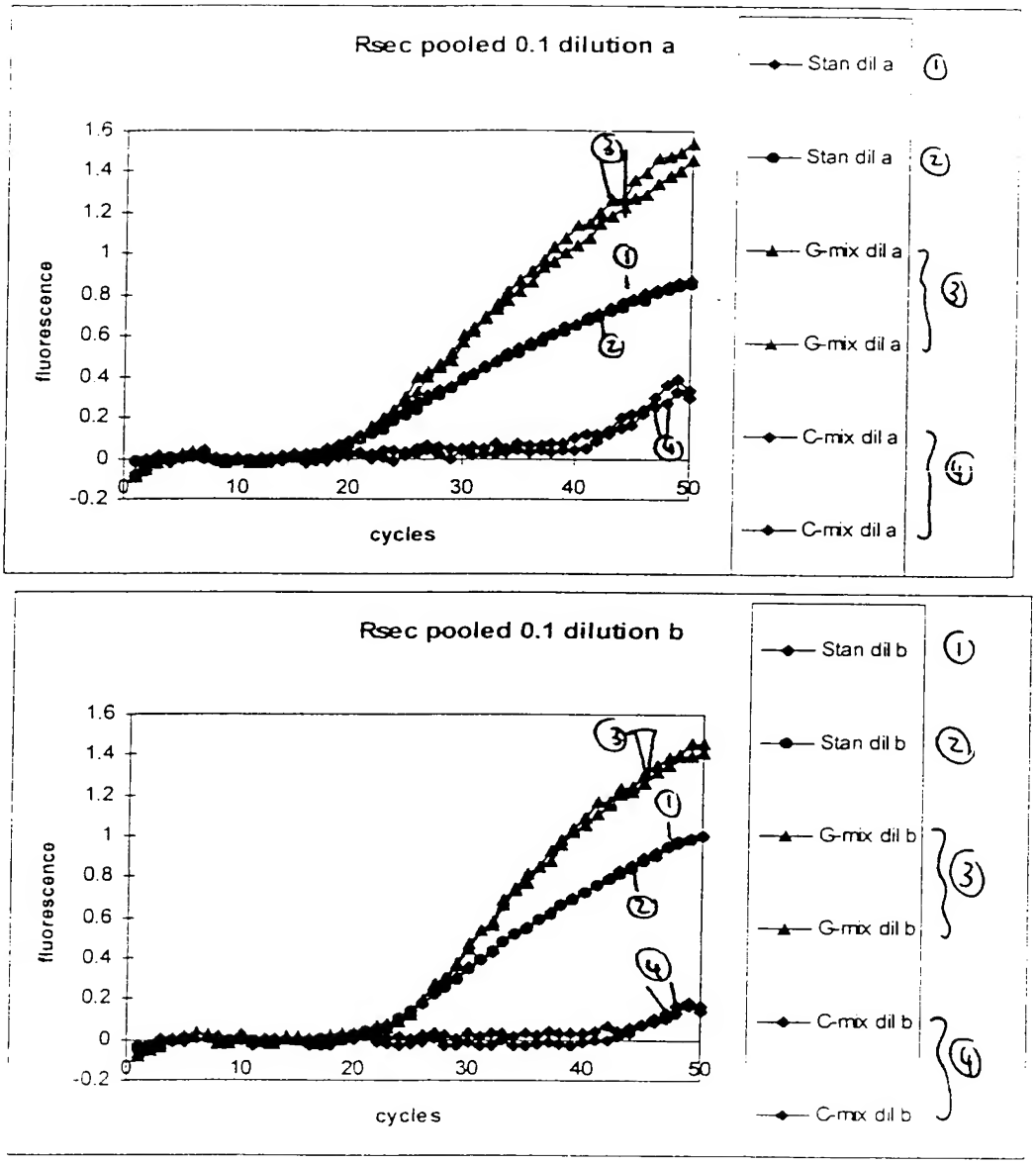


Figure 13 a and b

Preparation of *Pyrenophora teres* isolate K1916 for ARMS assay
(Selection in strobilurin analogue 2 medium)

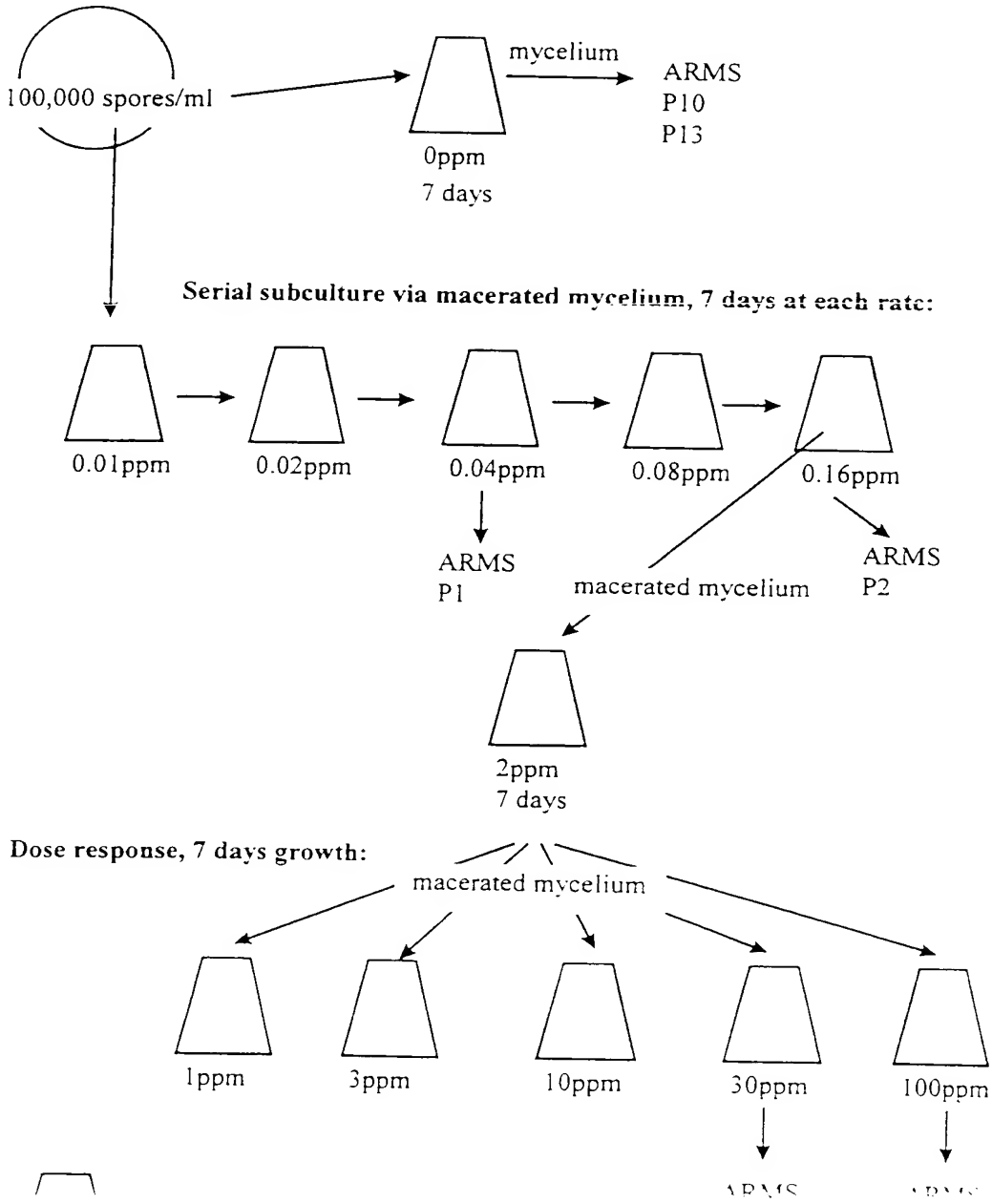


Figure 14a

Preparation of *Pyrenophora teres* isolates for ARMS assay
(Selection in strobilurin analogue2 medium)

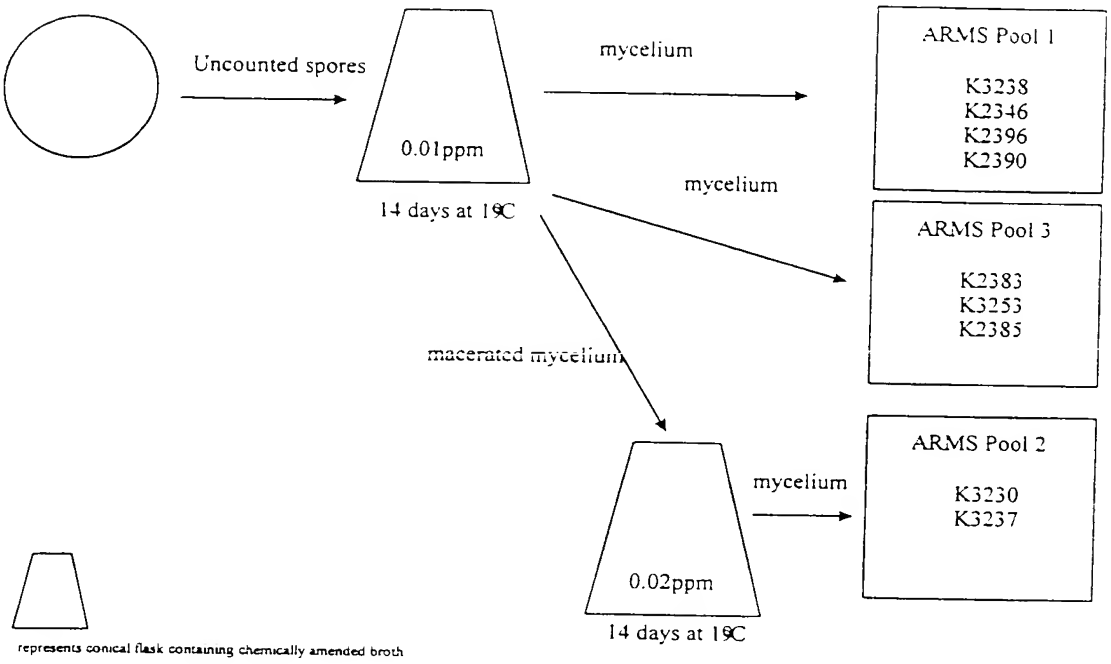
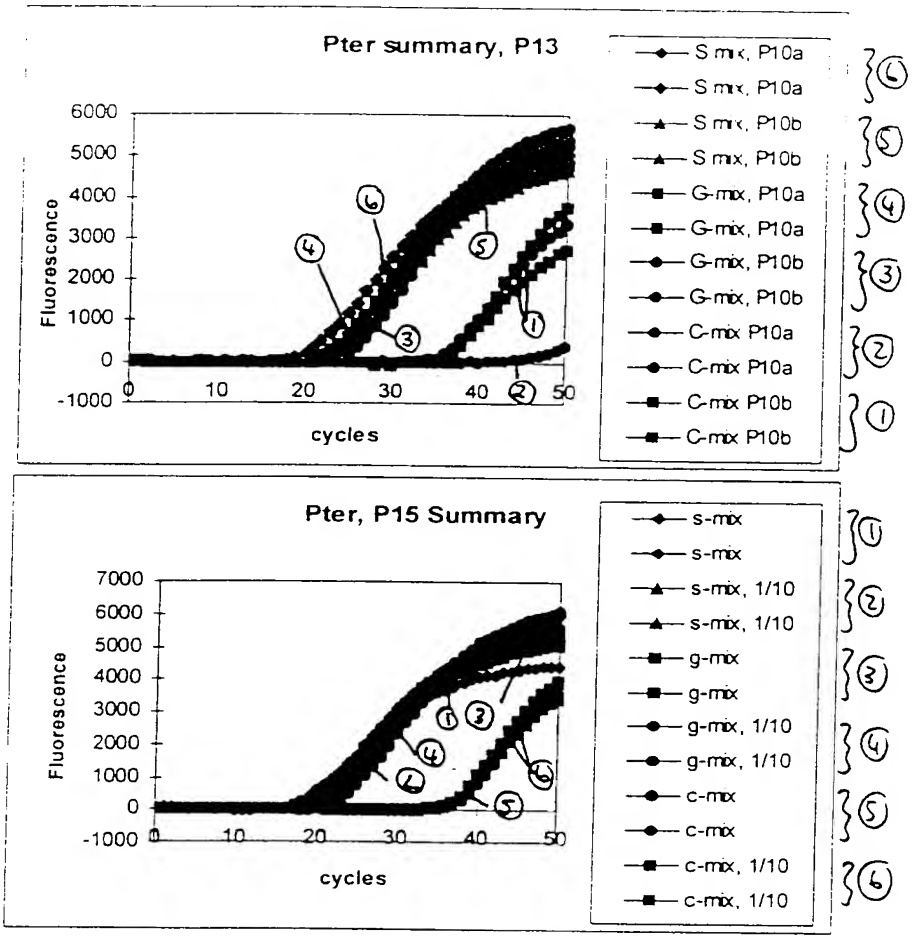
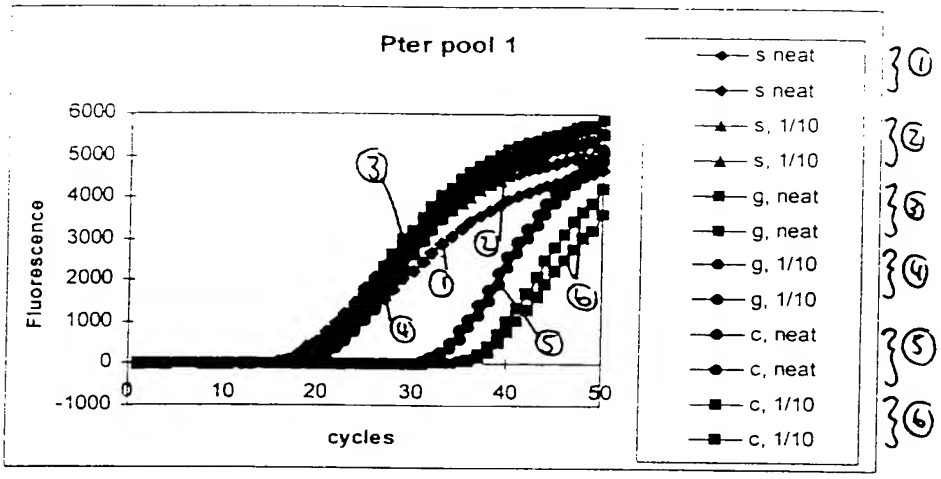
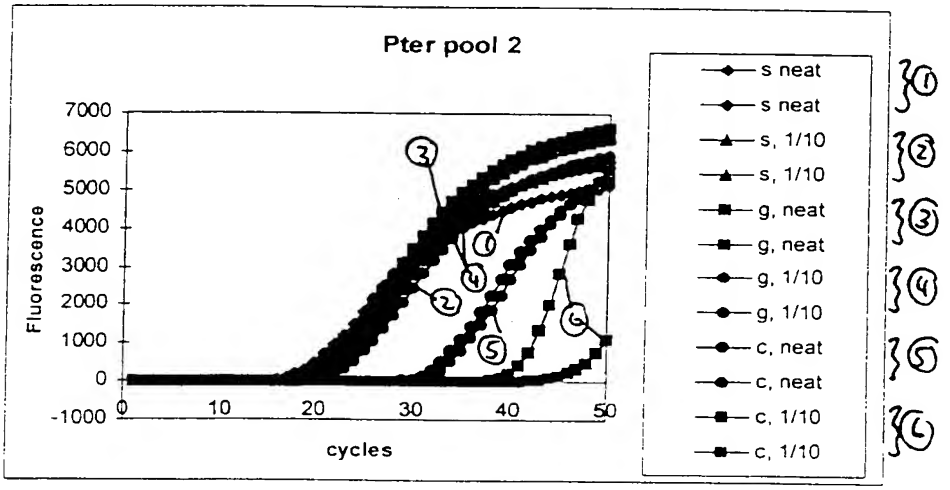


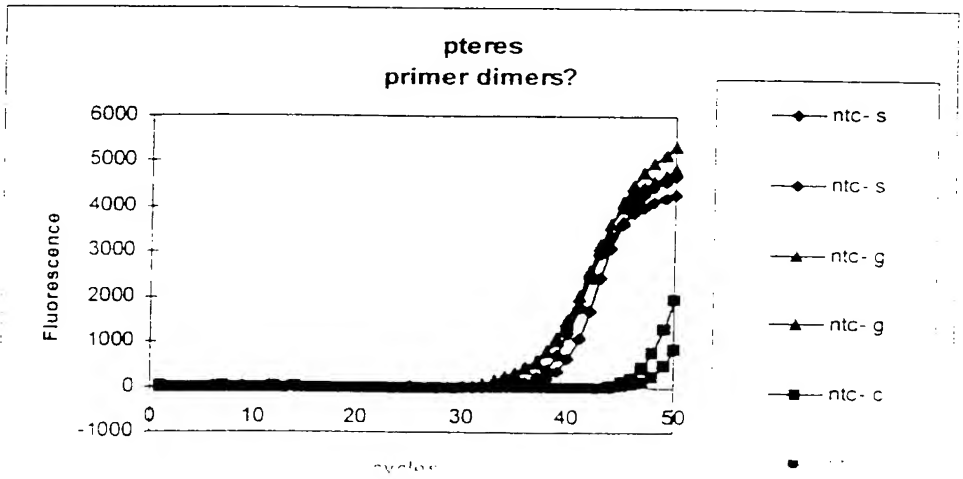
Figure 14b



Figures 15 a and b


Pter pool 1
Fluorescence
cycles
6000
5000
4000
3000
2000
1000
0
-1000
0
10
20
30
40
50
s neat
s neat
s, 1/10
s, 1/10
g, neat
g, neat
g, 1/10
g, 1/10
c, neat
c, neat
c, 1/10
c, 1/10
1
2
3
4
5
6


Pter pool 2
Fluorescence
cycles
7000
6000
5000
4000
3000
2000
1000
0
-1000
0
10
20
30
40
50
s neat
s neat
s, 1/10
s, 1/10
g, neat
g, neat
g, 1/10
g, 1/10
c, neat
c, neat
c, 1/10
c, 1/10
1
2
3
4
5
6


pteres
primer dimers?
Fluorescence
6000
5000
4000
3000
2000
1000
0
-1000
0
10
20
30
40
50
ntc- s
ntc- s
ntc- g
ntc- g
ntc- c